GROWTH BEHAVIOUR AND CHEMOSENSITIVITY OF SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK

A XENOGRAFT STUDY IN VIVO AND IN VITRO

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To my mother
To Ayke, Sidhi, Dhani

CONTENTS

CHAPTER I.

HEAD AND NECK SQUAMOUS CELL CARCINOMAS

1. Incidence and therapy

Squamous cell carcinomas are the most common malignant tumours of the head and neck. Head and neck cancer accounts for approximately 4% of all malignant neoplasms, with an estimated 42,900 new cases and 12,300 deaths in the United States of America in 1989 (1). In the Netherlands, the incidence rate according to the European standard population is 11.4, 1.6 and 0.9 per 100,000 population per year for the larynx, tongue and oropharynx in males and 0.6, 0.5 and 0.3 in females, respectively (2). Malignant neoplasms of the larynx account for 2.5% of all the tumours in males. The death rate in males is 3.0 and 3.1 per 100,000 population per year for laryngeal cancer and oral cancer, respectively (1,2).

Good therapeutic results for head and neck cancer with limited extension can be obtained either by surgery or radiotherapy alone. For the early stage of laryngeal cancer (T1) a 5 year actuarial survival rate of about 90% can be attained (3) and for carcinoma of the tongue up to 70%. The overall 5 year survival rate for all the stages together is about 40-50% for both laryngeal and tongue cancer (4).

Despite many efforts to improve the treatment results by combining different treatment modalities, the figures have remained virtually the same over the past 15 years. For some sites and stages it has been possible to reduce the local recurrence rate, however the overall survival rate has not increased because of an elevated rate of distant metastases and second primaries (5-7).

Chemotherapy which is known to improve the survival rate for many types of cancer, such as testicular cancer, Hodgkin's disease, non-Hodgkin childhood lymphomas, acute lymphocytic leucemia and childhood sarcomas (8-13), has been used increasingly in patients with locally advanced head and neck cancer, especially in cases with recurrent and metastatic disease, which cannot be controlled by surgery and/or radiotherapy.

The four most active and most frequently used agents for the treatment of head and neck squamous cell carcinomas are cisplatin, bleomycin, methotrexate and 5-fluorouracil. The overall response rate of these agents used as single agent therapy varies from 15 to 80% (14-16). With a combination of drugs Vogl et al. (17) found an increase in the response rate from 35% for methotrexate alone to 48% for a combination of methotrexate, bleomycin and cisplatin. An overall response rate of 70% has been reported for the combination of cisplatin and 5-fluorouracil in recurrent head and neck cancers (18) and 93% for previously untreated advanced squamous cell carcinomas of the head and neck (19). It has become clear that the tumour site and histopathology have a definite influence on the response rate, but that similarities in tumour location and histopathology by no means assure that different patients will show similar responses to anticancer drugs. It would be a great step forward therefore to be able to predict a patient's individual response to chemotherapy.

A general problem of cytotoxic drug treatment is the lack of reliable test systems which can predict the sensitivity of individual tumours to various drugs.
The development of experimental models for studying the biological behaviour of malignant tumours and especially models for chemosensitivity testing have received a great deal of attention for a long time and both in vitro and in vivo short-term and long-term assays have been introduced. An important prerequisite for testing tumours outside the patient is the preservation of the biological properties of tumour cells in both in vivo and in vitro systems.

2. In vivo models

Researchers have been attempting to perform xenotransplantation of tumours for more than 50 years. By using immunologically privileged sites for heterotransplantation such as the anterior chamber of the eye of rabbits and guinea pigs, it has became possible to grow human tumours in experimental animals (20). Unfortunately, the anterior chamber of the eye is small and therefore poses serious limitations. To overcome these problems, tumours have been transplanted intracerebrally (21), but the main disadvantage of this method is that the grafts are not easily accessible for inspection. The hamster cheek pouch appears to offer a more appropriate site where tumour grafts can be inspected and followed fairly easily (22). To abrogate the possible influence of an immune response which eventually develops, irradiation and or cortisone treatment can be applied (23-25).
In addition to these privileged sites, embryos and newborn animals have been used. In these animals, the immune system is still incompletely developed. However, the later aquired immunocompetence induces rejection of the grafts (26-29).
Apart from these animal models where graft rejection is mitigated or retarded, animal models where the immune system of the host is artificially suppressed have been introduced. Various methods have been applied to suppress the immune system: whole body radiation (30), whole body radiation plus cortisone (23), a combination of thymectomy, whole body radiation and bone marrow reconstitution (31), thymectomy and antilymphocyte serum (32) or thymectomy, cytosine arabinoside and whole body radiation (33). Although these models have produced satisfactory results the procedures are very time-consuming and long-term studies cannot be done because the induced immunosuppression is only temporary.

An important breakthrough in the field of in vivo tumour models was the discovery by Flanagan (34) of a nude mouse mutant with an autosomal mode of inheritance, which was found to be athymic (35). The lack of a functional thymus-dependent immune system allows the transplantation of human tumour cells into these animals without the risk of rejection.
The first successful transplantation of a human colon carcinoma into these nude mice was performed by Rygaard and Povlsen in 1969 (36). Since then a growing number of reports have appeared on the behaviour of human malignant tumours xenografted into nude mice (37-39). Nearly all types of tumour have been xenografted with varying degrees of success. It has been established that the take rate largely depends on the tumour type and site. A remarkably low success rate has

been reported for the transplantation of some solid tumours also of squamous cell carcinomas of the head and neck region (40,41).
The available data obtained from different tumours demonstrate that the biological and biochemical properties of the parent tumours are generally retained during serial passaging in nude mice (38,40,42-48).
Tumour xenografts in the nude mouse can guarantee a continuous source of the same tumour tissue for studying cell characteristics and kinetics. Furthermore, this model offers the possibility of studying the effects of cytotoxic drugs in a systematic way.
Good correlations have been reported between the response of human tumour xenografts to cytotoxic drugs and the patients response for several human malignancies (49-52).
The suitability of this model for testing cytotoxic drugs on squamous cell carcinomas of the head and neck has been shown by Sneeuwloper and Lindenberger (53) and by Braakhuis et al. (41). However, the latter authors also reported that methotrexate, a most powerful drug against squamous cell carcinomas, gave only a minimal response in the nude mice, which did not correlate with the sensitivity of the source tumours (41).
Although observations have shown that within certain limits the nude mouse is an attractive model for testing cytotoxic drugs, this model does not have any practical significance for predicting the chemosensitivity of individual patients. A major limitation is the duration of testing, which usually requires a period of about four months for fast growing tumours and more than one year for slow growing tumours.

A more rapid in vivo method for cytotoxic drug testing, the subrenal capsule (SRC) assay, was introduced by Bogden et al. (54,55) in the late seventies. This method involves transplanting small pieces of solid tumours into the subrenal capsule of immunocompetent mice and then exposing them to various cytotoxic drugs. The difference between the volume of the treated and untreated tumours measured after 6 days is thought to be a reliable parameter for the effect of the drugs. The effect of a normal host versus graft reaction is assumed to be insignificant during this short period.
The value of this assay is still a matter of debate. Aamdal et al. (56) and Rajnay et al. (57) found that the chemosensitivity profiles of xenografted human tumours using the 6-day subrenal capsule assay were of the same ranking as those of subcutaneous transplantation in nude mice. However, a detailed histological evaluation of over 800 SRC grafts revealed an intense inflammatory reaction and fibrosis and the complete absence or only a minor amount of tumour tissue in the majority of the grafts after 6 days (58). This indicates that the drug sensitivity patterns obtained with this assay using only macroscopic criteria do not correlate with the actual degree of tumour regression.
The major drawback of the SRC assay therefore appears to be the difficulty in establishing whether the changes in the volume of the xenograft are due to changes in the number of tumour cells or to the inflammatory reaction.

3. In vitro models

Apart from the in vivo models, several methods for in vitro chemosensitivity testing of human tumours have been introduced. So far none have found general acceptance. The oldest method is the cytologic-morphologic assessment of the action of cytotoxic agents in cell-tissue cultures. The first short-term cultures of human and animal tumours as a test system for cytotoxic agents were performed by Gellhorn et al. (59) and Biesele (60). A better standardisation of the culture conditions was reported by Eagle and Foley (61,62). Various modifications of the method of testing cytotoxic agents have since been developed by introducing the inhibition of dehydrogenase activity and the incorporation of radioactively labeled nucleic acid precursors as parameters for establishing drug effects (63-69). Despite the large number of investigations, it is not yet clear whether these tests allow any reliable conclusions on tumour behaviour.

In 1977, Hamburger and Salmon (70) introduced an in vitro assay which measures the reproductive capability of tumour cells in soft agar. This stemcell assay has been widely advocated as a method for culturing human tumours and for prospectively selecting the most appropiate anticancer drug for an individual patient. In retrospective and prospective clinical studies on ovarian cancer and multiple myeloma, Salmon et al. (71) reported true sensitivity and resistance predictions of 92% and 100% respectively. In a review by Salmon the positive prediction rate varied from 60% to 92% and the negative prediction from 83% to 100% with an overall percentage of 71% and 91% (72).

The main disadvantage of this method is that the proportion of human tumours which show clonal growth is frequently less than 50%. Von Hoff reported that from more than 8,000 tumours cultured only 31% had sufficient in vitro growth for drug testing and only 8% of the specimens with adequate growth demonstrated in vitro sensitivity to currently available antineoplastic drugs. This agrees with the data reported by Rozencweig et al. (74). They stated that only 34% of solid tumours were suitable for drug testing.

Squamous cell carcinomas of the head and neck have also been tested using the clonogenic stemcell assay. However, because of the very low plating efficiency, Mattox et al. (75) concluded that the clonogenic assay does not contribute to the management of patients with cancer of the head and neck.

Improvement of the plating efficiency of head and neck squamous cell carcinoma was obtained by passaging the tumours first in athymic mice, but for some cytotoxic drugs there was no correlation between the in vitro and in vivo sensitivity tests (76).

A second disadvantage which can seriously interfere with the final results of this stemcell assay is the technique used for disaggregation of the tumours. Irrespective of the methods used, the tumour cell preparations invariably contain aggregates of tumour cells and such pre-existing cell aggregates are difficult to differentiate from proliferative cell colonies (77). In addition, Bijman et al. (78), in their study on head and neck squamous cell carcinomas, reported that each dispersal technique , either mechanical, enzymatic, chemical or a combined procedure, could yield typical cell suspensions which contained relatively different types and numbers of cells and

that this may influence the results of the in vitro drug sensitivity testing.
Summarizing the difficulties associated with obtaining true single cell suspensions and the low plating efficiency for many tumour types, preclude the routine application of human tumour colony forming assay for chemosensitivity testing (72,77,79-82).
For in vitro chemosensitivity testing of tumour cell-lines several rapid colorometric assays have been described. In recent years, the tetrazolium (MTT) assay , has been the most widely used method (83, 84). This method is based on the ability of living tumour cells to reduce a tetrazolium-based component (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a blue formazan product. However, the MTT assay for drug sensitivity testing of primary tumour samples is limited because the contaminating normal cells may also reduce the tetrazolium. Therefore it only offers a valid and simple method of assessing chemosensitivity in established cell lines (83). The sulforhodamine B protein stain (SRB) assay for in vitro chemosensitivity testing provides a better linearity with cell number and a higher sensitivity than the MTT assay. In contrast to the MTT assay, the SRB assay stains recently lysed cells and the cell debris is not stained, as was reported by Skehan et al. (85) and Keepers et al. (86).

4. Purpose of this study

In view of the difficulties encountered with the in vitro models, the use of nude mice as an in vivo model for growing and testing human tumours may have more clinical relevance than culturing disaggregated single tumour cells in an artificial environment.
In this study, some characteristics of the nude mouse as a model for studying human head and neck squamous cell carcinomas are further explored.
Chapter II deals with the take rate and growth characteristics of human squamous cell carcinomas. In chapter III the stability of the DNA content of these tumours was studied during serial passaging with the use of DNA cytometry. In chapter IV the value of this model for testing cytotoxic drugs is analysed using growth curves, histology and the DNA profile as parameters. In chapter V a method is introduced for the production and incubation of thin tumour slices of equal thickness for cytotoxic drug testing. In chapter VI the reliability of two different in vitro chemosensitivity tests, is investigated.

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CHAPTER II.

GROWTH CHARACTERISTICS OF HEAD AND NECK SQUAMOUS CELL CARCINOMAS IN NUDE MICE

Part of this chapter has been published in: Eur J Cancer Clin Oncol 22: 1211-1222, 1986 by: Elprana D, Kuijpers W, van den Broek P and Wagener DJTh.

1. Abstract

Growth behaviour of human squamous cell carcinomas from the head and neck region was studied in nude mice. Tumour growth was observed in 10 out of 13 tumours transplanted, including 2 lymph node metastases. The take rate percentage related to the number of tumour inocula used, varied from 10 to 90% in the first passage and from 70 to 100% in the subsequent passages. No significant difference could be established in the growth pattern, except from the first to the second passage. Histological studies demonstrated preservation of the original histopathological features, invasion of two tumour lines into the fibrous capsule, but no distant metastasis. Comparison of growth curves with histological features demonstrated that growth curves are not a reliable measure for the number of vital tumour cells present.
The time needed for the tumour to obtain a diameter of about 4 mm proved to be a reliable parameter for the determination of the growth characteristics. It is concluded that both growth curves and histology are indispensable when growth behaviour of xenografted tumours is studied.

2. Introduction

Since the first successful transplantation of a human colon carcinoma in athymic nude mice (1), many studies dealing with xenotransplantation of a large variety of malignant tumours in this animal model have been reported (2-4).
These tumours present during repeated passages in this animal a histopathological picture, which appears very similar to that of the original tumour (3,5,6), although the degree of differentiation may show some variation (3,4,6,7). In addition, the human origin of the growing tumour cells has repeatedly been demonstrated by chromosome analysis (8). The degree of success in establishing a tumour line varies considerably between various tumour types and appears to be largely dependent on the degree of cellular differentiation and whether tumours are used from the primary site, from metastases or from recurrent tumours (2,4,5,9-12).
Only limited data are available on the transplantability and growth behaviour of squamous cell carcinomas of the head and neck region in the nude mouse (10,13-18). These studies reveal a low take rate, varying between 26 and 40% for primary transplants and of about 50% for subsequent passaging. Although the factors responsible for this invariably low take rate are not fully understood, it has been discussed that the degree of differentiation (10,15) and the possibility of bacterial contamination of the primary grafts (10) play an important role.
However, Wennerberg et al. (15) failed to find a significant difference in take rate between specimens taken as non-sterile biopsies and as sterile excisions. Growth characteristics of these tumour xenografts have only been determined from measurements of tumours grafted subcutaneously and hardly any data are available which compare growth curves and histological features.
This report deals with transplantation of a series of head and neck squamous cell carcinomas into nude mice with special reference to primary take rate, serial passaging and histology as well as to the interrelationship between histological

features and growth curves.

3. Materials and Methods

3. 1. Transplantation

Tumour tissue was obtained from fresh surgical specimens of 11 previously untreated squamous cell carcinomas from different primary sites in the head and neck region and 2 lymph node metastases. A solid piece of tumour measuring about 1 cm^3 was dissected from the surgical specimen, under sterile conditions, immediately after removal from the patient. Since the surface of the tumour is always contaminated, the specimens were dissected from the deeper parts of the tumour and the necrotic tissue was removed. Further cleansing was achieved by rinsing the tumour tissue three times in cold balanced Hank's solution (4°C). The tissue was subsequently stored in this solution until use.

Transplantation was performed within 1 hr of tumour excision. For inoculation of the tumour into the nude mouse, the tissue block was cut into pieces of about 1-2 mm^3 and these were implanted subcutaneously in the flank of the nude mice (female Balb/c, nu/nu, age 7-12 weeks, obtained from TNO-breeding unit, Rijswijk, The Netherlands). The mice were kept in cages with filter caps and provided with sterile bedding, food and drinking water containing Terramycin (250 mg/l). The ambient room temperature was 26°C and the air humidity 70%. For serial transplantation, the tumour graft was removed under ether anaesthesia and the same procedure was followed as for the primary transplants. The number of mice used for each tumour varied between 10 and 20.

Tumour growth was measured with the use of a Vernier caliper at weekly intervals, and biweekly in the case of rapidly growing tumours. Tumour volume was calculated as 0.5 x the product of the three dimensions and was plotted on a linear scale (19).

3. 2. Microscopy

After varying survival times, the mice were sacrificed with ether and the entire tumours dissected. The animals were inspected for the presence of metastases. For light microscopy, fragments from the original tumours, the entire xenografts and the draining lymph nodes were fixed in phosphate buffered (0.1M; pH 7.4) formaldehyde(4%), dehydrated in graded alcohols and embedded in paraffin or glycol methacrylate. Sections (3-7 μm) were stained either with haematoxylin-eosin, toluidin blue, methylgreen pyronin or Masson-Goldner for keratin.

For electronmicroscopy, small tumour fragments were fixed in phosphate buffered (0.1M; pH 7.4) glutaraldehyde (2%) for at least 4 hours. After postfixation in 1% osmium tetroxide in phosphate buffer (0.1M; pH 7.4) for one hour, the specimens were rinsed in buffer and after dehydration in graded alcohols embedded in Epon (20). Ultrathin sections were contrasted with saturated aqueous solution of uranyl acetate (21) and subsequently with lead citrate (22). The sections were examined with a Philips EM 300 electronmicroscope.

4. Results

4. 1. Take rate

A total number of 11 primary squamous cell carcinomas and two lymph node metastases were transplanted. The sites of origin, the TNM- and the histopathological classification are shown in Table 2.I. Growth of the primary tumour was achieved in 9 out of 11 cases corresponding to 82%. From the two transplanted lymph node metastases only one appeared to grow. Tumour lines were subsequently established from all the growing tumours (100%). The passage levels are indicated in Table 2.I. The take rates, i.e. the proportion of tumour grafts of a single tumour resulting in growth in the first and subsequent passages, are summarized in Table 2.II. These data show a low primary take rate of the inocula for seven out of 12 tumours. This take rate increased considerably sometimes to 100% in subsequent passages.

4. 2. Growth curves

Growth curves of transplants from a single tumour revealed a large variation. This was true not only for the primary transplants, but also for the subsequent passages as shown in Fig. 2.1 for tumour HN-5. The variation in the growth curves could not be related to the size of the inoculum, since it appeared that transplantation of tumour fragments of different size failed to show any clear correlation between the size and type of growth curve.

In order to study the possiblity as to whether selection of fast and slow growing population of tumour cells might be the underlying cause of the observed variation of the growth curves, fragments of tumours with a slow and an accelarated growth pattern were transplanted. Both resulted in a comparable variation of the growth curves. Because of this large individual variation of growth curves obtained from one tumour, these curves could not be used as a reliable parameter for calculating the growth rate in the various passages. The duration of the initial lag-phase, i.e. the time needed for the tumour to obtain a general mean diameter of about 4 mm, was therefore chosen as the parameter for growth.

The initial lag-phase appeared to be the largest for the first passage and to decrease in the higher passages to a rather stable value, as determined for six tumours (Table 2.III). The initial lag-phase for the first passage appeared to vary between 3.6 and 9.8 weeks, with the exception of tumour HN-7 which showed the extra-ordinarily high value of 33 weeks. In further passages, the initial lag-phase varied between 1.1 and 5.1 weeks. No significant difference was found for this value between the primary tumour HN-2 and its lymph node metastasis HN-2M.

4. 3. Light- and electronmicroscopy

Histological examination of the transplanted tumours demonstrated preservation of the original histopathological characteristics in all passages studied (Figs. 2.2, 2.3 and 2.4). Only tumour HN-7 showed a slight dedifferentiation. Metastasis to lymph nodes or other organs was not observed in any of these lines. Throughout the observation period all tumours were surrounded by a fibrous capsule containing many capillaries. Because of the limited number of tumours available from

Table 2.I. Head and neck squamous cell carcinomas transplanted into nude mice.

Localization	Stage	Histology	Passage
1. Supraglottic (HN-1)	T1N2M0	Poorly diff., keratinizing	No growth
2. Epiglottic (HN-2)	T2N1M0	Mod. diff. keratinizing	10th
3. Lymph node met.(HN-2M)**	T2N1M0	Mod. diff., keratinizing	10th
4. Epiglottic (HN-3)	T4N1M0	Poorly to mod. diff., keratinizing	No growth
5. Piriform sinus (HN-4)	T2N1M0	Mod. diff., keratinizing	10th
6. Tongue (HN-5)	T2N0M0	Mod. diff., keratinizing	5th
7. Tongue (HN-6)	T2N0M0	Mod. to well diff., keratinizing	6th
8. Epiglottic (HN-7)	T1N0M0	Well to mod. diff., focallykeratinizing	3rd
9. Tongue (HN-8)	T1N0M0	Mod. diff., non keratinizing	3rd *
10. Supraglottic (HN-9)	T3N0M0	Mod. to well diff., keratinizing	4th
11. Piriform sinus - Lymph node met.(HN-10)	T1N1M0	Mod. to well diff., keratinizing	No growth
12. Tongue (HN-11)	T2N1M0	Mod. diff., keratinizing	3rd *
13. Tongue (HN-12)	T2N0M0	Poorly to mod. diff., non keratinizing	2nd *

Overall primary take rate 10/13 (77%). Take rate primary tumours 9/11 (82%).

* Terminated at indicated passage. ** Lymph node metastasis of HN-2.

Table 2.II. Percentual take rate (number of takes in relation to the number of mice transplantated). For the first passage 10 mice were used. For serial passaging the number of mice varied between 10 and 20.

	Passages									
Tumour	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
1. HN-1	0(0/10)									
2. HN-2	30(3/10)	80(8/10)	100(10/10)	100(20/20)	85(17/20)	85(17/20)	100(20/20)	100(20/20)	100(20/20)	100(20/20)
3. HN-2M*	10(1/10)	30(3/10)	86(12/14)	100(18/18)	100(20/20)	90(18/20)	100(17/17)	100(10/10)	100(10/10)	90(18/20)
4. HN-3	0(0/10)									
5. HN-4	10(1/10)	80(8/10)	92(12/13)	93(14/15)	80(16/20)	80(12/15)	85(17/20)	70(14/20)	100(10/10)	75(15/20)
6. HN-5	100(10/10)	100(15/15)	93(14/15)	100(8/8)	100(8/8)					
7. HN-6	30(3/10)	100(10/10)	87(13/15)	100(20/20)	80(16/20)	80(12/15)				
8. HN-7	10(1/10)	90(9/10)	100(10/10)							
9. HN-8	20(2/10)	90(9/10)	90(9/10)							
10. HN-9	20(2/10)	20(2/10)	100(10/10)	100(10/10)						
11. HN-10	0(0/10)									
12. HN-11	90(9/10)	60(6/10)	80(8/10)							
13. HN-12	80(8/10)	90(9/10)								

* Lymph node metastasis of HN-2.

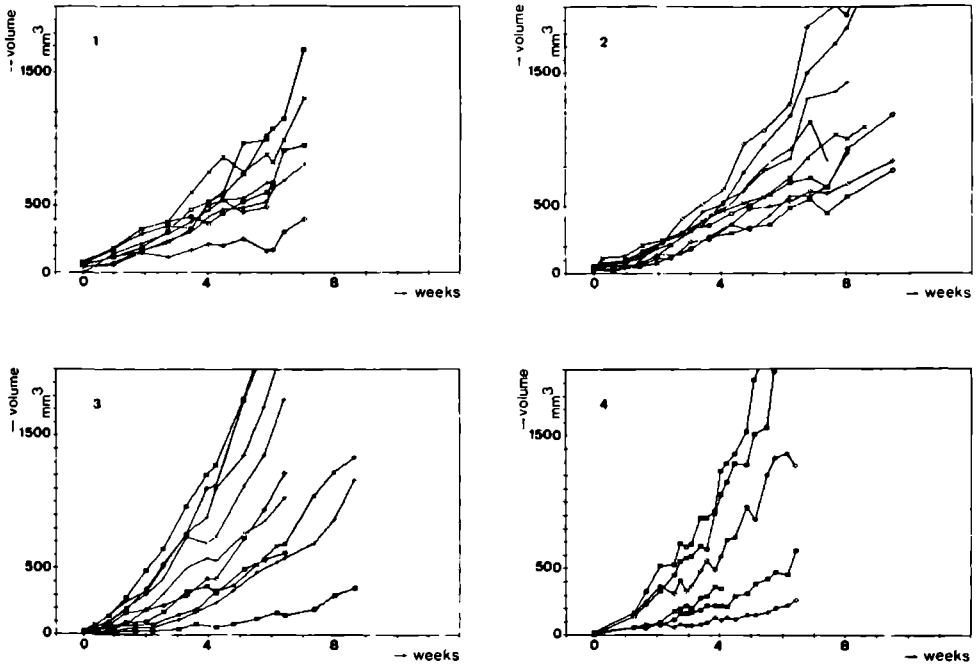


Figure 2.1. Growth curves of tumour 5 (HN-5) during the first four passages. Growth curves of tumours which were used for histological studies, during the first 5 weeks are not indicated.

Table 2.III. Initial lag phase of six established head and neck squamous cell carcinoma lines. Initial lag phase (in weeks ±S.D.; n: number of tumours measured) is defined as the time needed to obtain a general mean diameter of about 4 mm.

	Passages									
Tumour	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
1. HN-2	7.0 ± 0.50 (n=3)		3.48 ± 0.63 (n=10)	3.52 ± 1.67 (n=20)	3.17 ± 1.24 (n=17)	3.24 ± 2.15 (n=17)	2.60 ± 0.08 (n=20)	3.02 ± 1.20 (n=20)	2.86 ± 0.53 (n=20)	3.77 ± 1.74) (n=20)
2. HN-2M*	7.4 (n=1)		5.10 ± 0.20 (n=12)	4.53 ± 1.63 (n=18)	3.45 ± 1.57 (n=20)	4.35 ± 1.70 (n=19)	3.01 ± 1.11 (n=17)	4.05 ± 2.54 (n=10)	3.80 ± 0.01 (n=10)	3.20 ± 0.92 (n=18)
3. HN-4	9.8 (n=1)	4.30 ± 0.80 (n=8)	3.65 ± 2.00 (n=12)	2.46 ± 0.70 (n=10)	2.67 ± 1.16 (n=14)	2.05 ± 0.04 (n=10)	1.55 ± 0.81 (n=17)	1.36 ± 0.35 (n=14)	2.06 ± 1.01 (n=10)	2.47 ± 0.96 (n=15)
4. HN-5	3.6 ± 0.01 (n=10)	1.19 ± 0.06 (n=14)	1.80 ±0.08 (n=14)	2.10 ±0.10 (n=8)	1.97 ±0.18 (n=8)					
5. HN-6	9.10 ±0.02 (n=3)	1.08 ±0.30 (n=10)	2.68 ±0.71 (n=13)	3.81 ±1.28 (n=20)	3.70 ±1.05 (n=16)	2.90 ±0.98 (n=12)				
6. HN-7	33 (n=1)	3.13 ± 0.38 (n=9)	3.76 ± 1.85 (n=10)							

* Lymph node metastasis of HN-2

the primary passages, histology of tumour growth was mainly studied in the subsequent passages.

After 1 week, the implantation site showed scattered islands of proliferating tumour cells concentrated in the periphery of the tumour transplant and accumulations of extravasated erythrocytes, fibroblasts and capillaries (Fig.2.3). This area was separated from the surrounding host tissue by a layer of fibroblasts with numerous granulocytes and capillaries. After 2 weeks, the islands of tumour cells had grown together (tumour size about 2 mm^3), forming one tumour mass (Fig.2.4A). Subsequently, the tumour developed a distinct compartimentalization. The various compartments were separated by connective tissue septa containing capillaries (Fig.2.4 B,C.). These septa were lined by a basal layer of regularly arranged tumour cells containing numerous mitotic figures (Fig.2.4 C) These were also observed in the periphery of the tumour bordering the fibrous capsule.

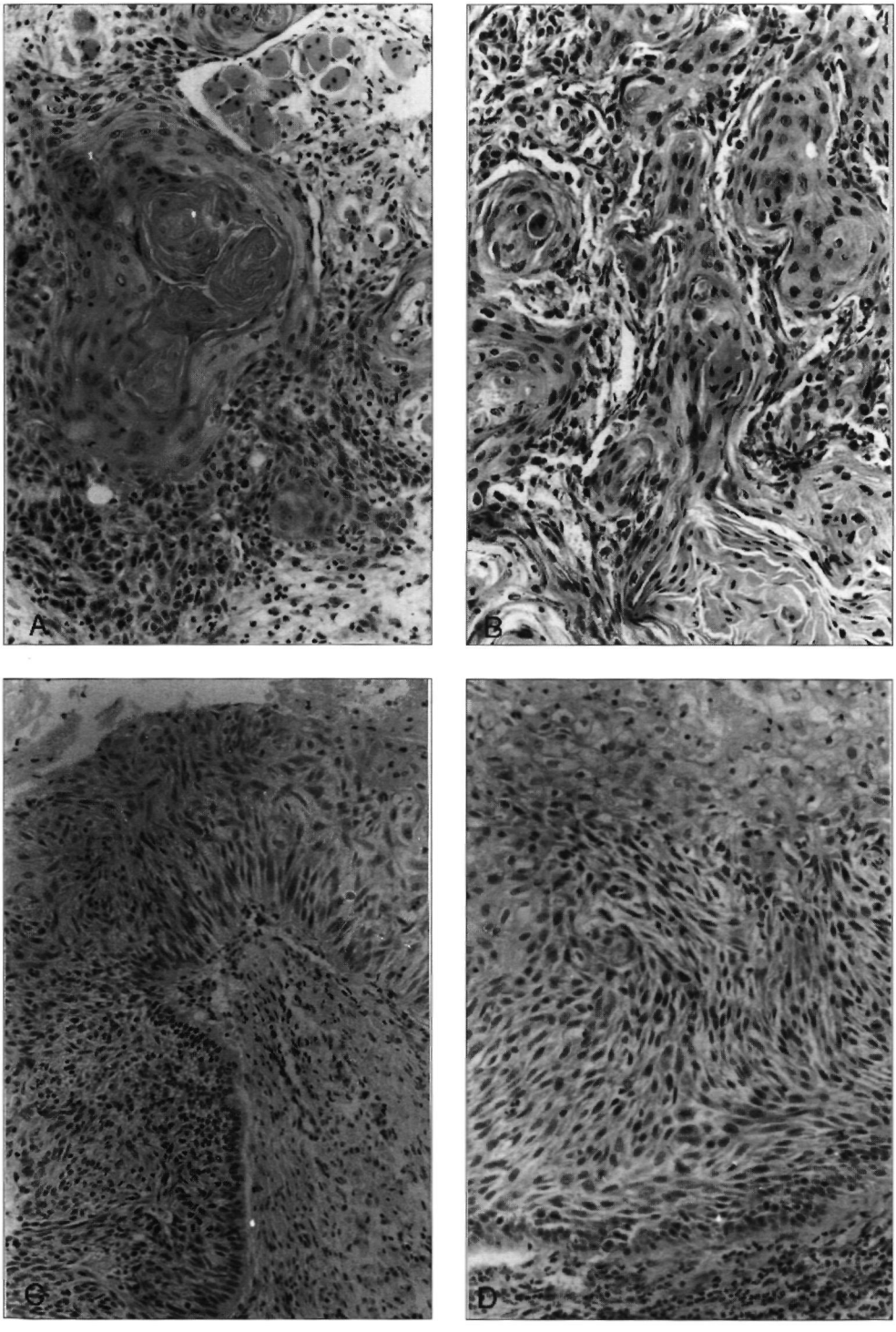
Electronmicroscopy of the xenografts showed a tumour mass surrounded by a desmoplastic connective tissue. The tumour was separated from the stroma by a basal lamina of a varying architecture, locally showing thickening, multilayering as well as discontinuities. The tumour cells revealed a largely varying number of desmosomes, however at some sites these intercellular connections were completely absent. The same was found for hemidesmosomes forming the connection between the tumour cells and the basal lamina (Figs.2.5; 2.6). Occasionally, processes of the tumour cells were found to penetrate through the basal lamina into the surrounding stroma (Fig.2.6 A,B). These phenomena were found in the different passages as well as in the primary tumours.

During further growth of the tumour, necrosis and/or desquamation became the most conspicious finding in the central parts of the tumour. This was often already marked at a tumour size of 30-50 mm^3 (Figs.2.7A; 2.8A,C). The presence of cellular debris, dyskeratosis and/or keratinization appeared to differ from line to line and was related to the degree of keratinization and the size of the tumours (Figs.2.7; 2.8). In all xenografts larger than 100 mm^3, the major part of the tumours consisted of debris, dyskeratosis and/or keratin lamellae (Figs.2.8B; 2.9A), sometimes showing a typical onion skin appearance (Fig.2.8B). In these larger tumours, the presence of tumour cells was limited to the peripheral part and the connective tissue septa, which often showed progressive degeneration (Figs.2.7C; 2.8D; 2.9).

Throughout the observation periods, invasion of tumour cells into the fibrous tissue was found in two tumour lines (HN-2 and HN-4) (Fig.2.10). This gave rise to the formation of satellite tumours, contributing to a lobular appearance. The earliest capsular invasion was already demonstrable after two weeks at a tumour size of 2 mm^3.

At a tumour size of more than 300 mm^3, an increasing number of tumours fluctuated on palpation and appeared to be filled with a viscous fluid (Fig.2.11). This was especially marked in the non-keratinizing tumours and those showing dyskeratosis.

Apart from differences in degree, this course of events was noticed in all tumour lines studied during various passages. Tumours which from their growth curves were classified as slow growing, revealed the same histological features as fast growing tumours after comparable survival times.


A
B
C
D

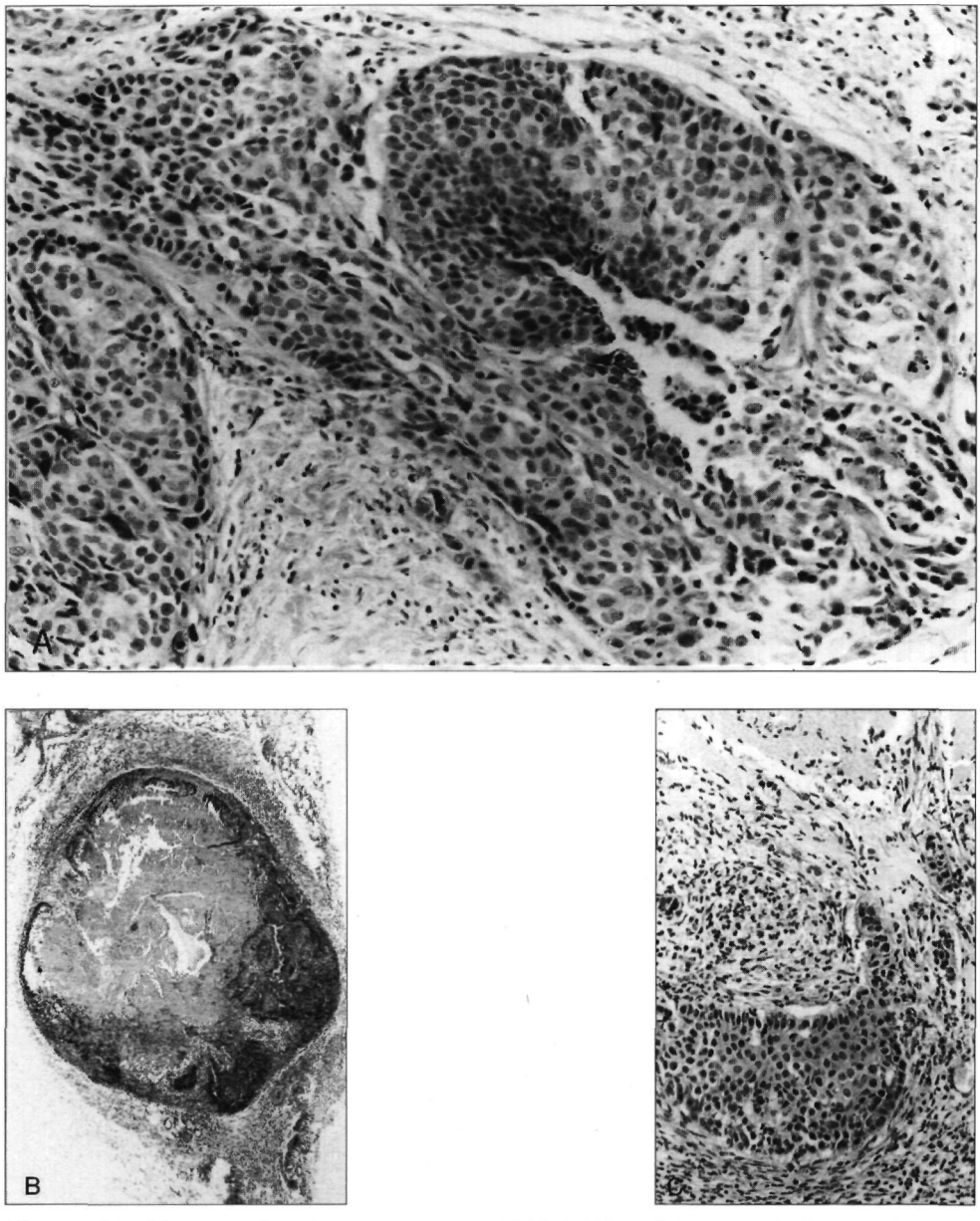


Figure 2.3. Micrographs of primary tumour HN-4 (A) and its growth characteristics in the nude mouse, after 1 week (4th passage; B: survey; C: detail) The site of implantation is surrounded by a fibrous capsule containing numerous inflammatory cells. Clusters of growing tumour cells are present in the peripheral part. The central part contains extravasal erythrocytes and fibrocytes (Haematoxylin-eosin, A,C: x 185; B: x 40).

← Figure 2.2. Micrographs of primary tumours (left) and their xenografts (right), showing preservation of the original histopathological characteristics: A: primary tumour HN-6; B: 4th passage. C: primary tumour HN-5; D: 3rd passage. (Haematoxylin-eosin; x 185).

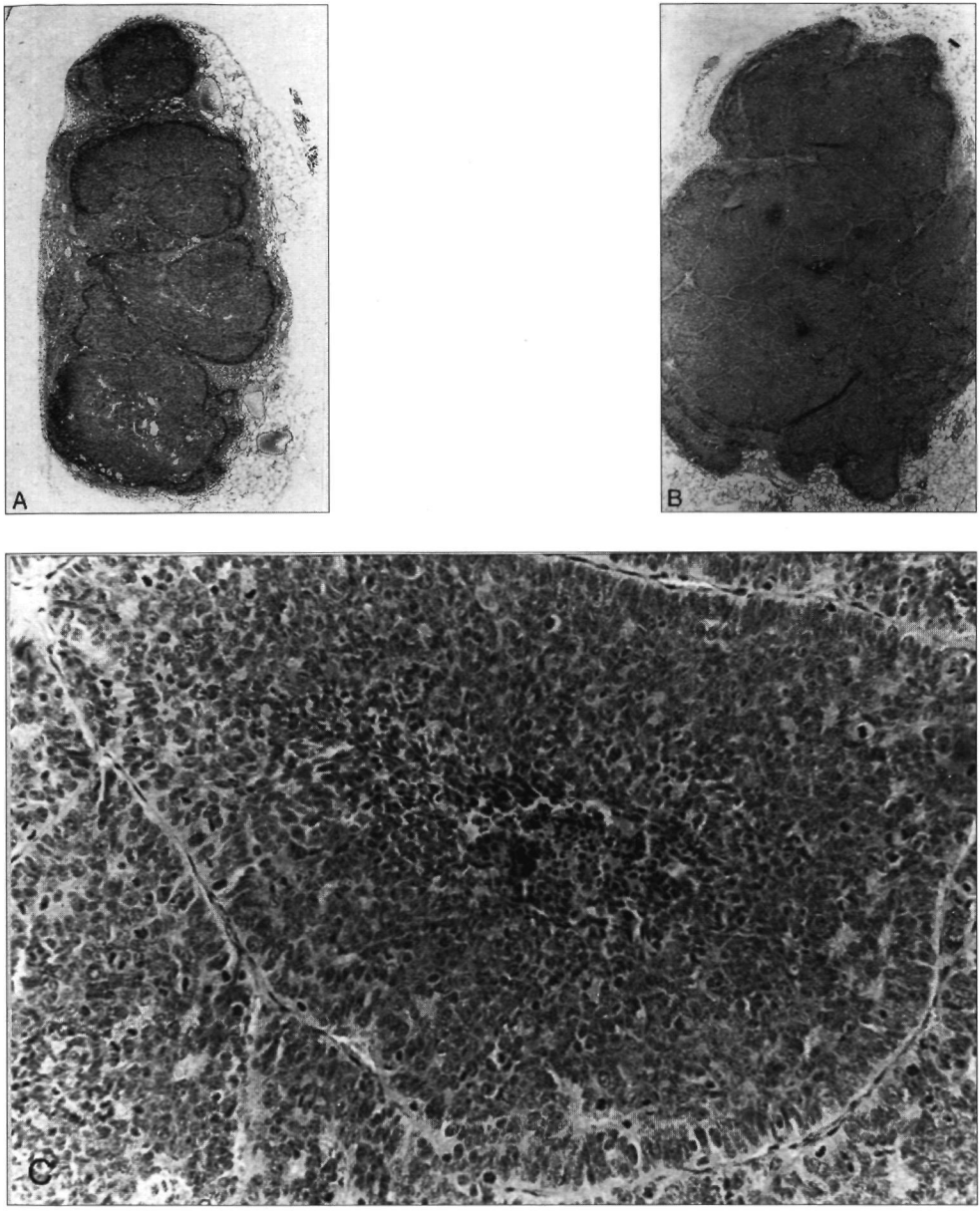


Figure 2.4. Micrographs of tumour HN-4 (4th passage), 2 (A; tumour size 2,0 mm³) and 3 weeks (B, C; tumour size 12.0 mm³) after xenografting. Fig. A shows encapsulated tumour mass with connective tissue septa of varying size in between. Fig. B shows one massive tumour, composed of various compartments. First signs of central necrosis are clearly visible at higher magnification in C (x 185) (Toluidin blue)

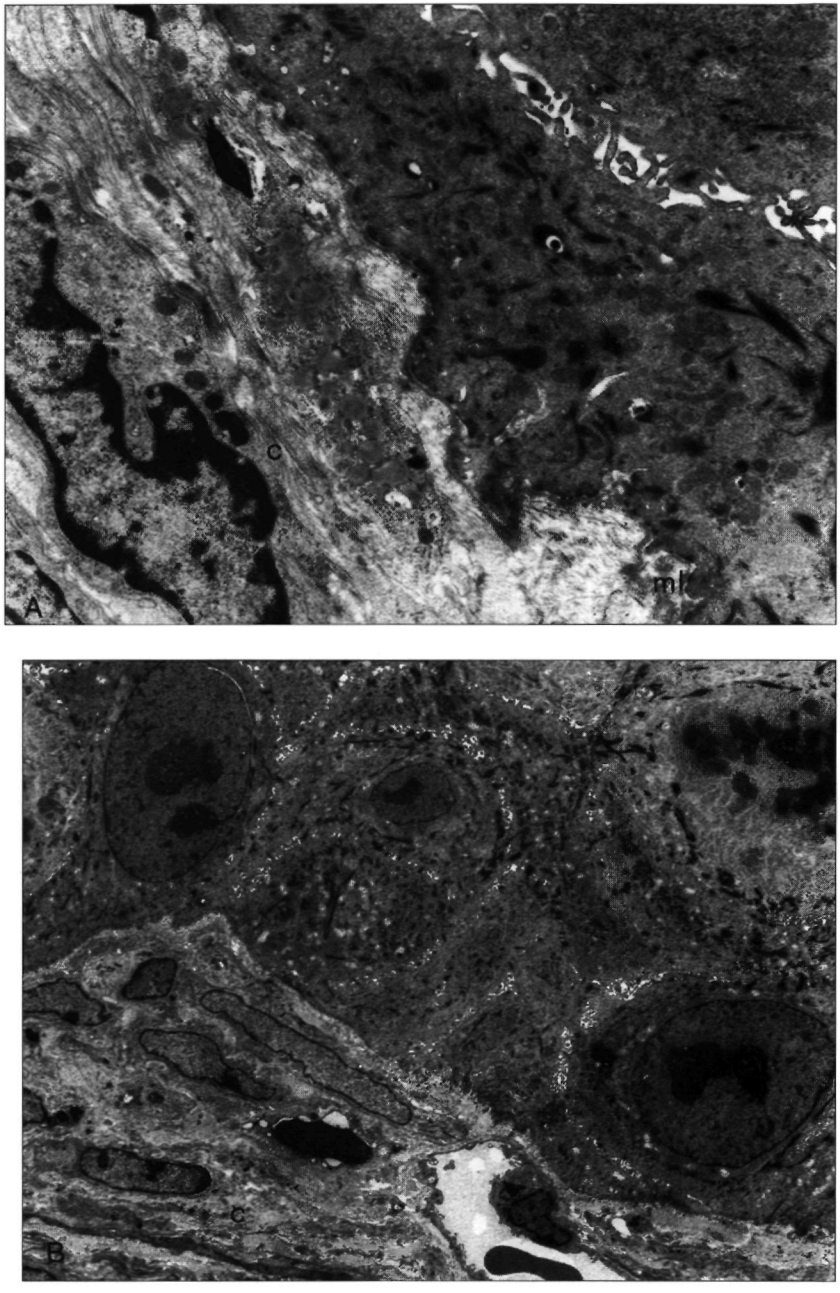


Figure 2.5. Electronmicrographs of xenografted tumour HN-2 showing local variation in the distribution of desmosomes and hemidesmosomes. Note multilayering (ml) of the basal lamina in A. c: fibrous capsule. (A: x 8,000; B: x 9,000).

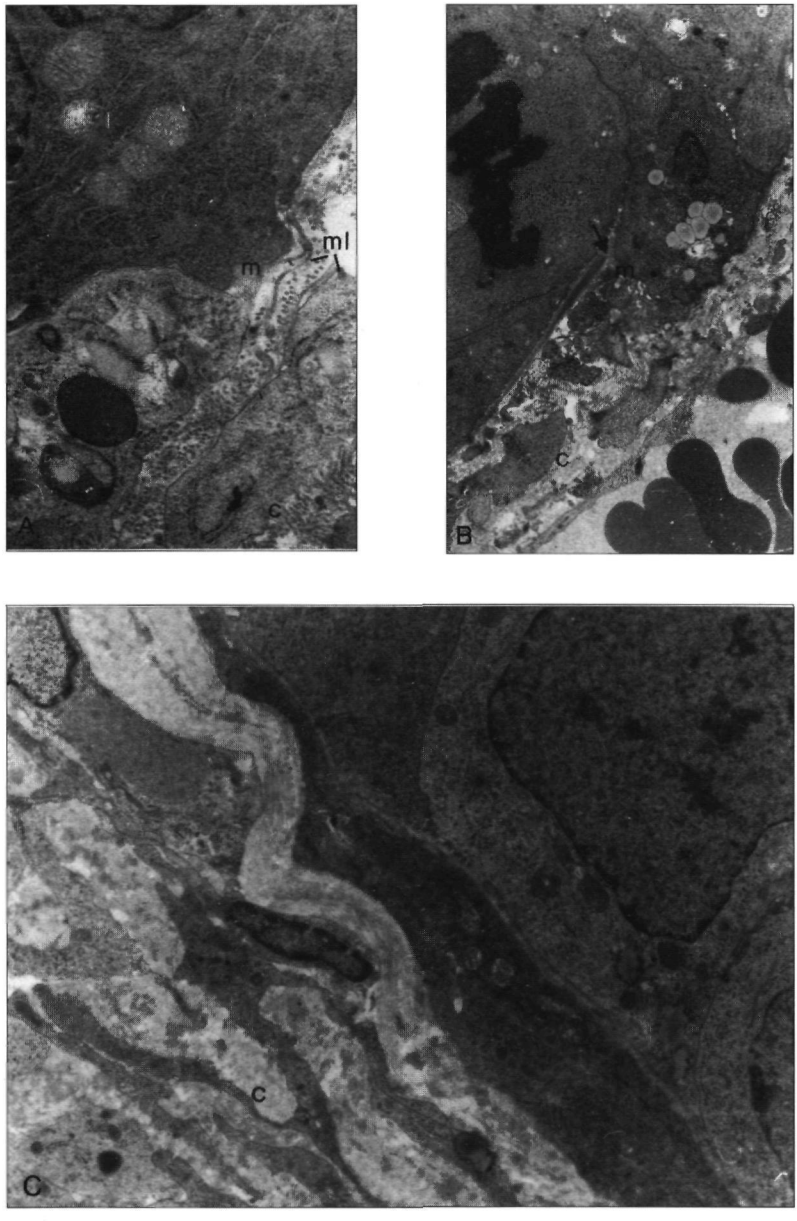


Figure 2.6. Electronmicrographs of xenografted tumours HN-2 (A) and HN-4 (B,C) showing microinvasion (m) of the tumour cells into the fibrous capsule (c). Note the complete absence of desmosomes and hemidesmosomes in A, B and C. (arrow: disruption of thickened basal lamina; (ml): multilayering of basal lamina; A: x 15,000; B: x 5,400; C: x 9,000).

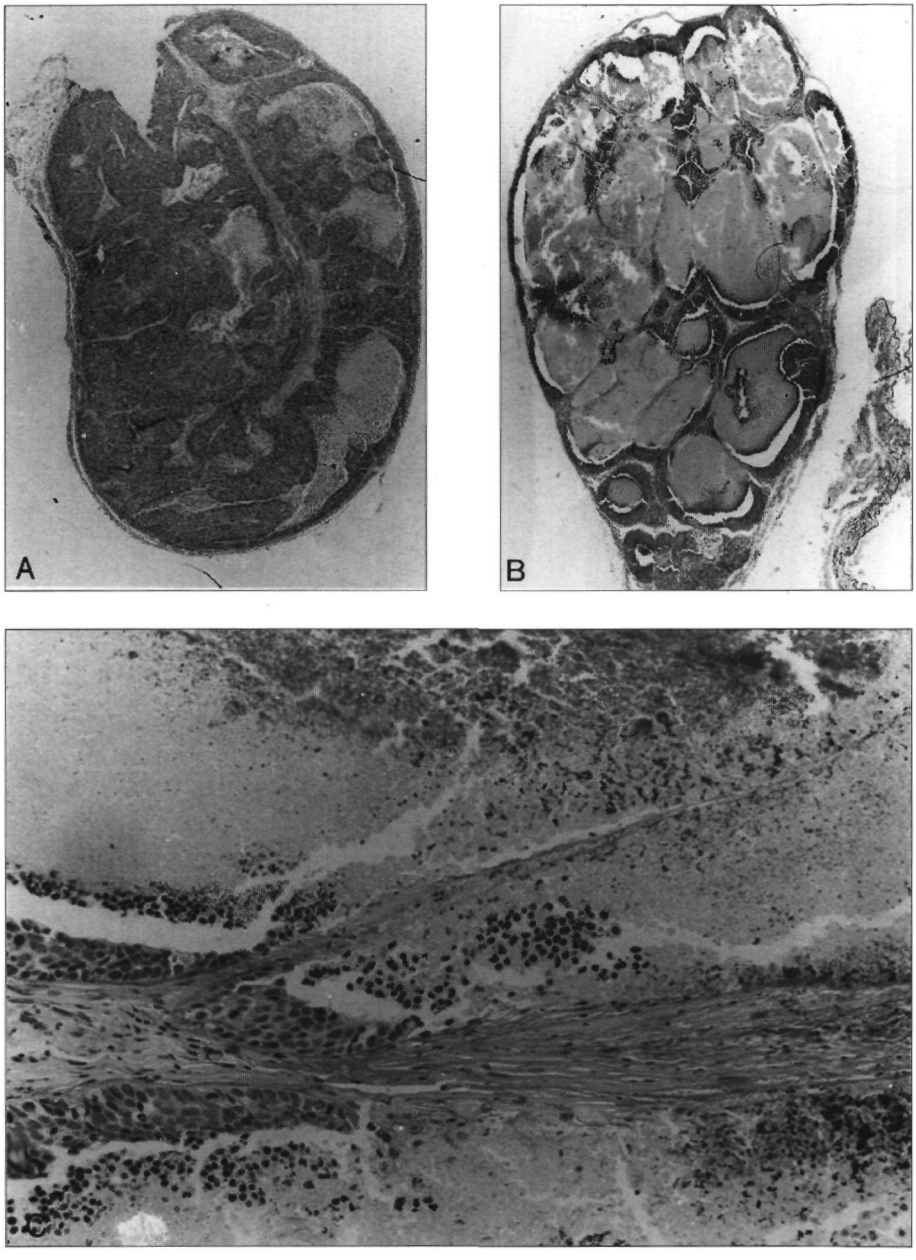


Figure 2.7. Micrographs of tumour HN-4 (5th passage), 4 (A; tumour size 40 mm³) and 5 weeks (B, C; tumour size 80 mm³) after transplantation, showing progressive central necrosis. By far the largest part of the tumour in B consists of dead material. Higher magnification (x 185) in C shows central degeneration of tumour and connective tissue septa. (Haematoxylin-eosin).

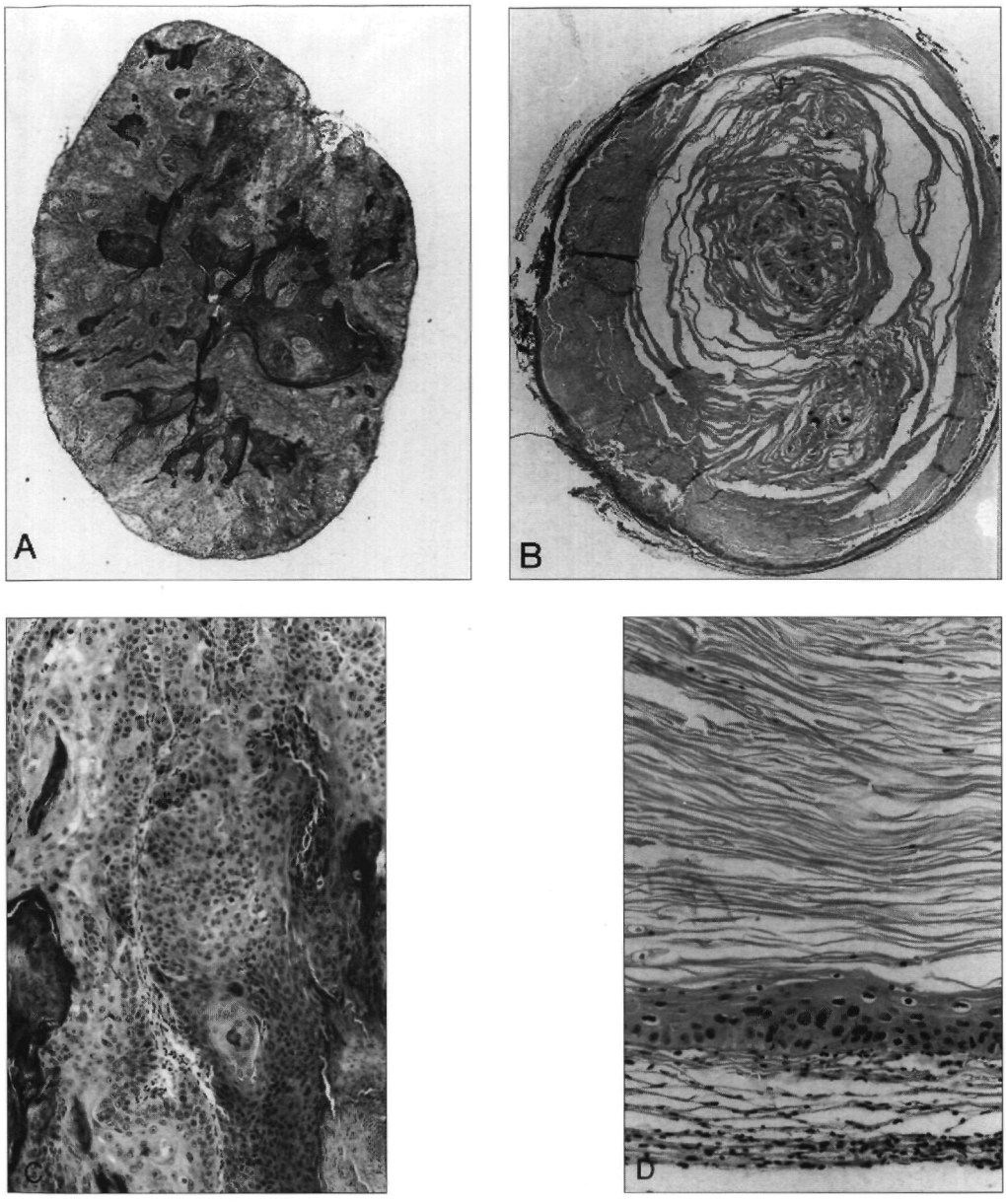


Figure 2.8. Micrographs of tumour HN-2 (8th passage) after 4 weeks (A, C; tumour size 50 mm³) and tumour HN-6 (2nd passage) after 6 weeks (B, D; tumour size 200 mm³). Note starting keratinization in the central parts of tumour HN-2 and the onionpeel appearance of the strongly keratinizing tumour HN-6. This tumour consists of a large mass of keratin, surrounded by a thin layer of vital tumour cells. (A, C: Masson-Goldner; B, D: Haematoxylin-eosin; C: x 90; D: x 185).

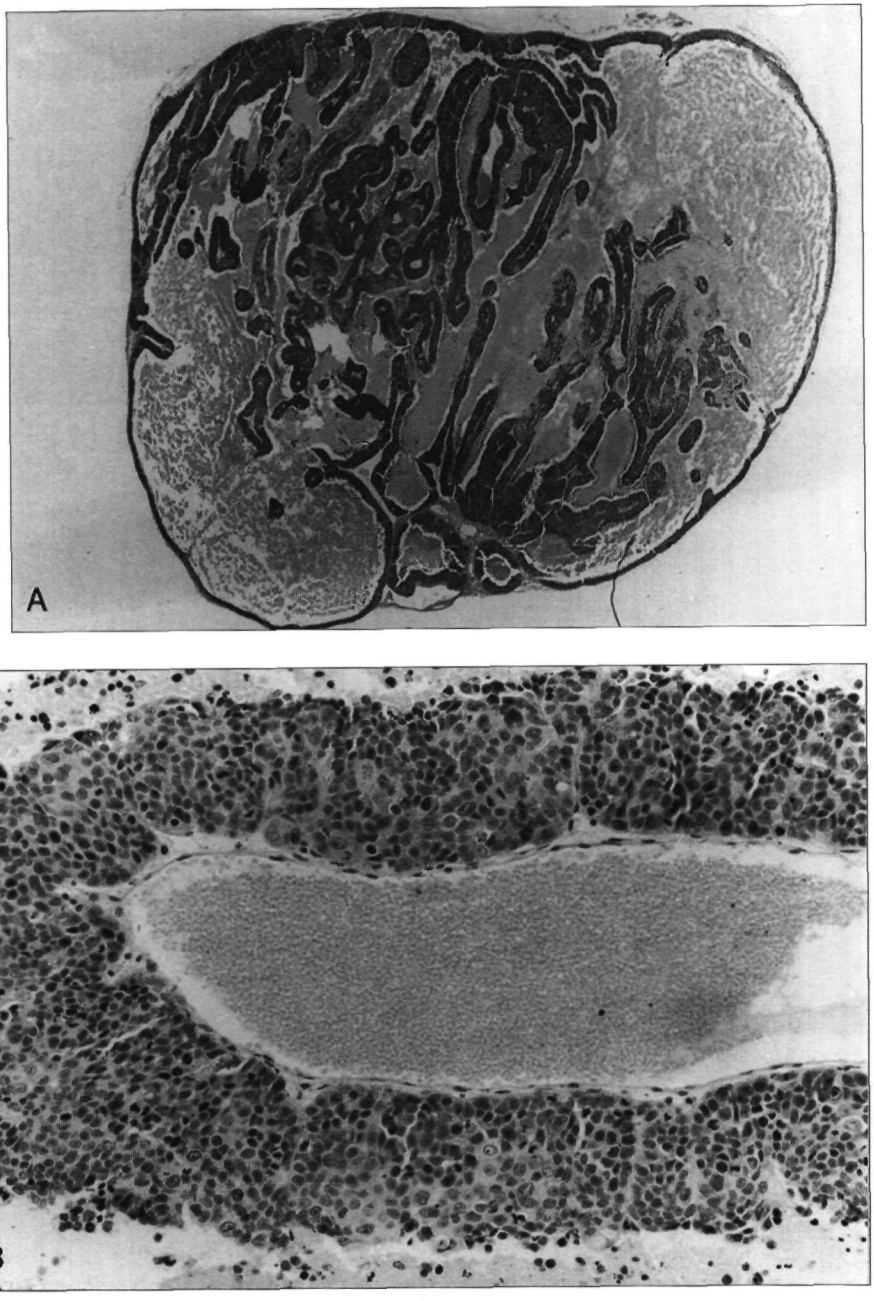


Figure 2.9. A. Micrograph of tumour HN-4 (4th passage) after 6 weeks (tumour size 300 mm³). Vital tumour cells are only present in the periphery and along the vascularized connective tissue septa. B. Higher magnification of septum with vital tumour cells and central capillary (Haematoxylin-eosin; B: x 185).

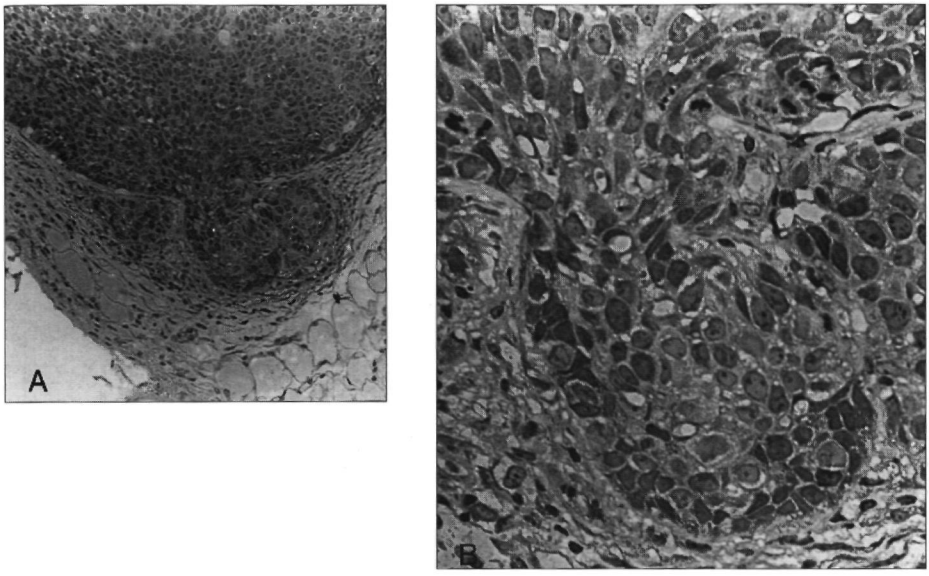


Figure 2.10. Survey and detail of invasion of tumour cells of tumour HN-4 into the fibrous capsule, 2 weeks after transplantation (4th passage). (Toluidin blue; A: x 125; B: x 400).

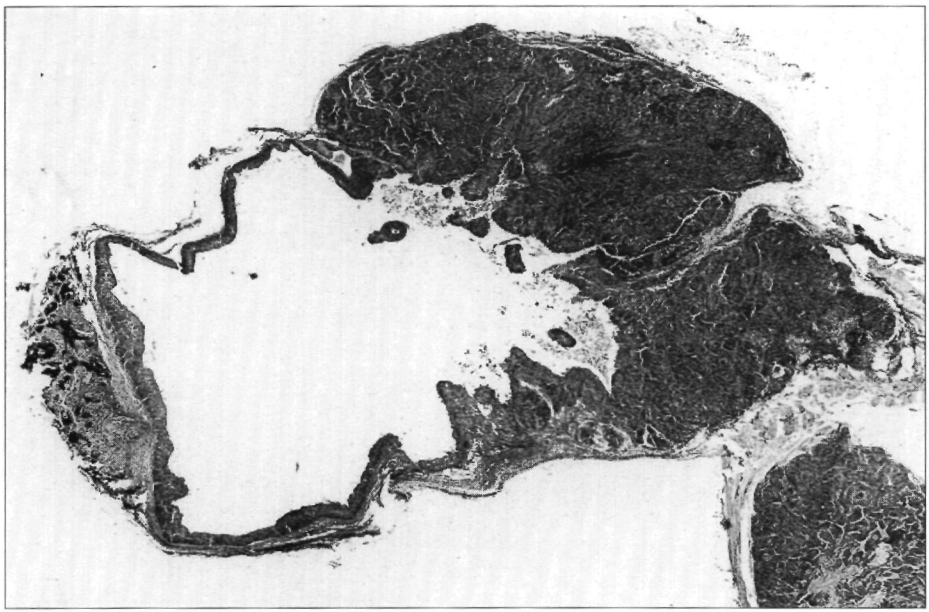


Figure 2.11. Micrograph of tumour HN-2 (6th passage; tumour size 500 mm³) showing cystic structure with central liquification. (Haematoxylin-eosin).

5. Discussion

The present study demonstrates that tumour lines of squamous cell carcinomas from the head and neck region can be established in nude athymic mice, generally retaining the original histopathological features of the donor tumours. No metastases to lymph nodes or other organs were observed. These observations generally agree with those made by other authors (10, 15,16), although both increased and decreased cellular differentiation have been described by Sharkey et al. (4,7) and Braakhuis et al. (10). Kyriasis et al. reported a lymph node metastasis of a human epidermoid carcinoma of the larynx in nude mice (23,24).

The transplants grew as circumscribed and encapsulated tumours without infiltration into the surrounding tissue, except for the invasion into the fibrous capsule. A comparable observation has been made in a human laryngeal epidermoid carcinoma by Kyriasis et al. (23).

In comparison with other studies on transplantation of squamous cell carcinomas of the head and neck region in nude mice, a high take rate of primary grafts and subsequent passages has been obtained in this study. This difference is difficult to explain, but the high take rate in this study (an overall take rate of 77% and 82% of primary tumours) demonstrates that head and neck carcinomas can be transplanted more successfully than has been described in previous experiments (10,15). It indicates that they do not belong to the category of solid tumours which are difficult to transplant successfully like gastric, mammary and genital tract tumours.

Because the suggestion has been made that poorly differentiated tumours have a better primary take rate than moderately and well differentiated head and neck squamous cell carcinomas (10,15), the degree of differentiation of the transplanted tumours might be held responsible for this difference in take rate. However, the present findings give no support to this, although the number of tested tumours is small. In this study a primary take rate of 82% was obtained chiefly with moderately to well differentiated tumours, while two out of the three tumours, which failed to take were poorly to moderately differentiated.

Also tumour site (10,15,25) tumour size, (15,25) malignancy grading (25) and the clinical course (26,27) have been suggested to influence tumour growth in nude mice. According to Wennerberg et al. (25) tumour size should be more important than the degree of differentiation. However, the small number of tumours of various categories tested in the present study and the absence of data on the clinical course do not permit to conclude on the existence of such a correlation.

A more acceptable explanation for the difference in take rate between the present study and those of others is likely to be found in the implantation technique, the number of inocula transplanted and the prolonged period of observation. Comparable suggestions have been made by Somers et al. (28). The data given in Table 2.II indicate that the number of primary takes with respect to the number of inocula used is often very low (sometimes only 1 out of 10). In the further passages the take rate increases considerably, sometimes reaching 100%. This is a confirmation of previous studies (10,29). A recent attempt to enhance the primary take rate of human squamous cell carcinoma of the head and neck in nude mice by total

body irradiation of the host failed to improve the take rate (30).
Determination of the growth rate of squamous cell carcinoma xenografts appeared to be difficult, owing to the large divergency between growth curves of the same tumour. This variation could not be attributed to the presence of fast and slow growing populations of tumour cells or to the size of the inocula, because initially the nutrition of the transplanted tumour is merely dependent on diffusion and therefore only the cells in the peripheral part will survive (Fig.2.3.B,C) (31). With a larger inoculum only the initial lag-phase will change.
Although host factors relating to the mitigated immune response and to the vascular supply of the tumours cannot be entirely excluded as possible explanations for the variation in growth curves, the histological observations suggest that accumulation of cellular debris and keratin lamellae within the tumour is a major contributing factor. This study also demonstrates that increase in the size of tumours beyond 50 mm^3 is influenced by cellular debris and desquamation products. This influence increases with the size of the tumour and shows individual variation. Calculation of growth rate of larger squamous cell carcinoma xenografts, from tumour doubling time is therefore an unreliable measure, because it is not related to the fraction of vital tumour cells, but chiefly to the amount of accumulated dead material. In view of these observations, the time needed for the tumour to obtain a general mean diameter of about 4 mm (initial lag-phase) proved to be a more reliable parameter in determining tumour growth characteristics. During this period the contribution of dead material to the size of the tumour is negligible.
This approach revealed a rather long initial lag-phase for primary transplants, decreasing in the subsequent passages to a limiting maximum value, and varying between different tumour lines. A comparable difference between the growth rate of the primary transplant and subsequent transplants has also been established by other authors for various other human tumour lines (8,11,32).
These data together with the difference in the take rate for primary transplants and following transplants, are suggestive of an adaptation of the tumour to the changed environmental conditions in the host which is assumed to result in an increase in the growth fraction as well as a reduction in the cell loss factor in subsequent passages (33-35).
The decrease in passage time observed between first and subsequent passages corresponds with an increased mitotic activity from first to second passage reported by Fu and Steel for rat mammary tumours (35) and with the increase in S-phase percentage cells in human lung tumours (36). An increased rate of ^{3}H-thymidine incorporation in tumour DNA during the second passage when compared with the first passage of head and neck squamous cell carcinomas, was reported by Wennerberg et al. (15). They suggested that this might be explained by a recruitment of G_o cells or by stem cell selection of clones of rapidly proliferating cells. This latter suggestion however seems to be in conflict with the observation of Lindenberger (37) who, using cytofluorometry, failed to find any change in DNA profiles in xenografts of squamous cell carcinomas from first to third passage.
Summarizing, this study demonstrates that squamous cell carcinomas from the head and neck region can be successfully established as lines in athymic nude

mice with preservation of their original histopathological features. Combined studies of both growth curves and histology are indispensable when investigating growth rate and growth behaviour, owing to the absence of a distinct relationship between tumour size and the amount of vital tumour tissue. This is of special importance when this model is used for evaluating the effect of various therapies.

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CHAPTER III.

DNA-CYTOMETRIC STUDIES ON XENOGRAFTS OF SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK

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1. Abstract

The primary tumours of four human head and neck carcinomas and their xenografts in nude mice were analysed with respect to their histological features and DNA ploidy level using DNA flow and DNA image cytometry. It was established that the histological characteristics and the DNA index of the xenografts did not differ from those of the parent tumours, whereas the growth rate remained stable during serial passaging. DNA flow cytometric analysis revealed the presence of both aneuploid and euploid cells in both parent tumours and their xenografts. During passaging the proportion of aneuploid cells increased. Analysis of the different cell populations by DNA image cytometry revealed the presence of aneuploid tumour cells and euploid host cells. This study demonstrates that the characteristics of the original tumours in terms of histological features and DNA ploidy are retained after xenografting.

2. Introduction

The biological and clinical relevance of in vivo studies on xenografted tumours largely depends on the preservation of the characteristics of the original tumours in the animal model.

Since the first successful transplantation of a human cancer in athymic nude mice by Rygaard and Povlsen (1) several characteristics have been studied on a large variety of tumour xenografts. From these studies it has become clear that, except for slight changes in the degree of histologic differentiation in some cases, the original histologic features of the human tumours including head and neck squamous cell carcinomas are retained during serial passaging in nude mice (2-10). The same holds true for the preservation of the human chromosome constitution (6, 11), the hormonal function (12-14) and other biochemical characteristics (15).

With respect to the cell kinetic characteristics of the xenografts, it has been established that the growth rate is generally stable over a considerable number of passages (10,16, 17). An increase in the growth rate during the first passages reported by some authors, has been attributed to an increased growth fraction and a reduced cell loss factor (18-21).

Also the cell cycle parameters are found to resemble closely these of the original tumour (22).

Although studies on the maintenance of the DNA ploidy status after xenografting are rather scarce, the available data from various tumours show that the DNA content generally remains stable during passaging in the nude mouse (23-25).

However the stability of this parameter has hardly been investigated in head and neck squamous cell carcinomas and the obtained results are controversial with respect to the preservation of the original clonal composition (7, 21).

As the cellular DNA content seems to be related to therapy responsiveness and prognosis (26-30), the preservation of this parameter is of crucial importance for making xenografted tumours a reliable model for drug sensitivity testing.

The purpose of the present study was to determine whether xenogeneic head and neck squamous cell carcinomas retain the DNA ploidy and histological features of

the original tumours after xenografting during serial passaging in nude mice.

3. Materials and Methods

Tumour tissue was obtained from fresh surgical specimens of previously untreated primary squamous cell carcinomas of the head and neck region and processed aseptically both for transplantation in nude mice and for measuring the DNA content by flow cytometry according to previously described methods (10, 31). Their site of origin and further characteristics are summarized in Table 3.I.

3. 1. Transplantation

For xenografting a piece of vital tumour tissue measuring about 1 cm^3 was dissected under sterile conditions from the deeper parts of the surgical specimen, immediately after removal from the patient. Subsequently the tumour tissue was rinsed thoroughly in cold (4°C) balanced Hank's solution. Transplantation was performed within 1 hour after tumour excision. For that purpose the tumour tissue was minced into pieces of about 1-2 mm^3 and 6-10 pieces were implanted subcutaneously over the flanks of 3-5 female Balb/c, nu/nu mice, age 8-10 weeks under ether anesthesia as described previously (10).

The remaining tumour fragments were used for flow cytometry and histological studies.

The mice were kept in cages with filter caps and provided with sterile bedding, food and drinking water. The ambient temperature was 26°C and the air humidity 70%. Tumour lines were maintained by retransplantation of regrown tumour xenografts in 3-5 mice in the same manner as with the primary tumours.

3. 2. Histology

For comparison of the histological features of the original tumours with those of the xenografts and for DNA image cytometry, the tumour tissue was fixed in phosphate buffered (0.1M) formaldehyde (4%, pH 7.4). After dehydration in graded alcohols the specimens were embedded in paraffin. For histological studies sections (7 μm) were stained with haematoxylin-eosin.

3. 3. Immunohistochemistry

For establishing the nature of the stroma in the xenografts a monoclonal antibody MECA-20 (kindly provided by Dr. A.M. Duijvestijn, University of Limburg, The Netherlands) directed against mouse endothelium was used (32).

For immunohistochemical staining tumour xenografts were frozen in liquid N_2 immediately after dissection from the mouse. Cryostat sections (7 μm) were placed on poly-l-lysine coated slides and dried at room temperature.

The sections were shortly fixed in cold aceton, rinsed in phosphate buffered saline (PBS, pH 7.4) and incubated with the monoclonal antibody MECA-20 (diluted 1:20 in PBS) for 60 minutes at room temperature. After rinsing in PBS, the sections were incubated with peroxidase conjugated rabbit anti-rat serum (diluted 1:50 in PBS) containing 20% normal mouse serum for 30 min., again rinsed in PBS and incubated with peroxidase conjugated swine anti-rabbit serum (diluted 1:50 in

Table 3.I. Squamous cell carcinomas of the head and neck region: tumour characteristics and patients survival.

Localization	Stage	Histology	DNA index (DI)	Survival (months)
A. Floor of the mouth (HN-13)	T2N0M0	Mod. to well diff., keratinizing	2.0	alive; 41
B. Epiglottis (HN-14)	T1N1M0	Mod. to well diff., keratinizing	1.8	alive; 41
C. Trigonum retromolare (HN-15)	T2N1M0	Mod. to well diff., keratinizing	2.2	died of relapse; 15
D. Floor of the mouth (HN-16)	T2N0M0	Mod. diff., keratinizing	1.65	alive; 24

PBS) as a second antibody containing 20% normal mouse serum. Following rinsing in PBS, peroxidase activity was detected by incubation in 0.05 M Tris-HCl buffer (pH 7.8) containing 1% 3,3 diaminobenzidin tetrachloride and 0.01% H_2O_2. The sections were rinsed in tap water, counterstained with Mayer's hemalum and embedded in DPX.

3. 4. DNA flow cytometry

For DNA flow cytometry the fragments obtained from the primary tumours and xenografts were washed twice in Hank's balanced salt solution and once in Eagle's minimal essential medium (MEM). After preincubation in MEM, containing collagenase (2 mg/ml, type II, 132 U/mg, Cooper Biomedical) and hyaluronidase (0.5 mg/ml, type I, 370 U/mg, Sigma), for 1 hour at 37°C under mild agitation, the tissue suspension was allowed to settle in the test-tube for 5 minutes and the supernatant containing mainly lymphocytes was subsequently discarded.

The residual tumour tissue was incubated with trypsin (0.25 mg/ml) and dithioerythritol (3 mg/ml) overnight at 4°C in Hank's balanced salt solution.

After centrifugation at 200 g, the pellet was resuspended in 4 ml MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Europe) and 100 µl RNase (2.5 mg/ml) and incubated for 30 minutes at 37°C under agitation.

Any undissociated tumour tissue was allowed to settle in the test-tube for 5 minutes and the resulting supernatant was spun down at 200 g. The cell yield was subsequently determined. After fixation using 70% ethanol, aliquots containing 10^6/ml cells were stained in phosphate buffered saline plus 1 mM EDTA containing propidium iodide (40 µg/ml, Calbiochem) and 100 µl RNase (1 mg/ml). The cell suspensions were filtered through a 50 µm nylon mesh, incubated for 10 minutes at 37°C and measured with FCM (Ortho Diagn, MA; system 30 H). Propidium iodide stained human lymphocytes were used for optical alignment of the FCM apparatus. The amplifier gain setting was adjusted until the stained human lymphocytes (diploid) fell in channel 100 on a 512 channel scale.

Generally 25,000 cells were measured per histogram. The coefficient of variation (CV) of the standard was approximately 2% and varied between 2 and 5.5% for the tumour cells. The CV was calculated as follows: Full width (mm) at half maximum peak height divided by distance (mm) of peak channel to origin of histogram. This is multiplied by 42.5.

The modal DNA content, expressed as a DNA index (DI), is the ratio between the peak channel number of the aneuploid peak and that of the euploid peak of the same sample.

From each passage 3-5 tumours grown in different mice were tested.

3. 5. DNA image cytometry

In addition to DNA flow cytometry, DNA image cytometry was applied on selected parts of the xenografts according to the method introduced by Oud et al. (33). This method which has been extensively described elsewhere (33-35), allows the analysis of the DNA content on paraffin-embedded tissues. In short, fifty micron sections of formaldehyde fixed, paraffin embedded xenografts were dewaxed and subsequently rehydrated. Guided by adjacent hematoxylin-eosin

Table 3.II. Take rate and initial lag phase of squamous cell carcinomas of the head and neck region in nude mice.

	Percentual take rate 1)		Initial lag-phase 2)	
Tumour	1st Passage	Subseq. Pass.	1st Passage	Subseq. Pass.
A. HN-13	60%	100%	3-4 weeks	1-2 weeks
B. HN-14	40%	80%	4-6 weeks	2-3 weeks
C. HN-15	40%	100%	4-5 weeks	1-3 weeks
D. HN-16	20%	60%	16 weeks	6-8 weeks

1) Number of takes in relation to the number of mice transplanted.

2) Time needed for the tumour to obtain a general mean diameter of about 4 mm.

stained 5 µm sections the tumour tissue was selectively dissected from the thick section, using a dissecting microscope and subsequently incubated in PBS (pH 7.4) containing 0.1% protease at 37°C for 60 minutes. Incubation was terminated by the addition of 5 ml cold (4°C) PBS. The isolated nuclei were washed twice in PBS by intermediate centrifugation steps and counted with a Coulter counter. About 30,000 nuclei were spun down and resuspended in 200 µl fetal bovine serum.
A monolayer smear was prepared on a glass slide using a cytocentrifuge, air-dried, and subsequently fixed in a mixture containing methanol, formaldehyde and acetic acid (85:10:5 by volume) for 1 hour.
The nuclei were stained with pararosanilin (36) and the DNA content of 200 stained nuclei was measured using the CAS 100 system (37). Normal human cells were used as a control.

4. Results

Four primary untreated squamous cell carcinomas of the head and neck region and their successfully transplanted tumour lines, passed to 3 and more subsequent passages, could be investigated. The characteristics of these tumours and their growth behaviour in nude mice are shown in Tables 3.I and 3.II
The take rate percentage related to the number of tumour inocula used, varied from 20 to 60% in the first passage and from 60 to 100% in the subsequent passages. The initial lag phase (the time needed for the tumour to obtain a general mean diameter of about 4 mm (10) varied between 3 and 16 weeks for the first passage and between 1 and 8 weeks in subsequent passages.
The primary tumours contained in addition to tumour cells a varying amount of stroma with inflammatory cells (Fig.3.1). The xenografts grew as lobulated tumours surrounded by a fibrous capsule. During serial passaging the amount of stroma gradually diminished. In the tumour mass, thin connective tissue septa containing capillaries were present (Fig. 3.1). Immunostaining of cryostat sections with the monoclonal antibody MECA-20 directed against mouse endothelium revealed a positive reaction in the vascular endothelium (Fig.3.2) indicating the mouse origin of the stroma. At a volume of more than 50 mm^3, the central areas of the tumours showed an increasing amount of keratin and/or cellular debris. The xenografts retained the basic characteristics of their original tumours, with respect to both type and grade. None of the tumours showed local infiltration or haematogenous spread.
The histological picture of the xenografts closely resembled the parent tumours, as is illustrated for tumour A in fig. 3.1. . DNA flow cytometry is a reliable and rapid method to establish the DNA profiles of the tumours during serial passaging.
The flow cytometric DNA profiles of the primary tumour and the xenografts until the sixth passage of tumour A, are shown in fig. 3.3. The primary tumour showed a large peak of euploid cells and a smaller peak of aneuploid cells.
The tumour xenograft showed a comparable histogram in the first passage. In subsequent passages, the tetraploid peak and the G2/M peak became more predominant while the euploid peak decreased. This persisted up to the sixth passage and no difference was found between the DNA index (2.0) of the primary tumour and

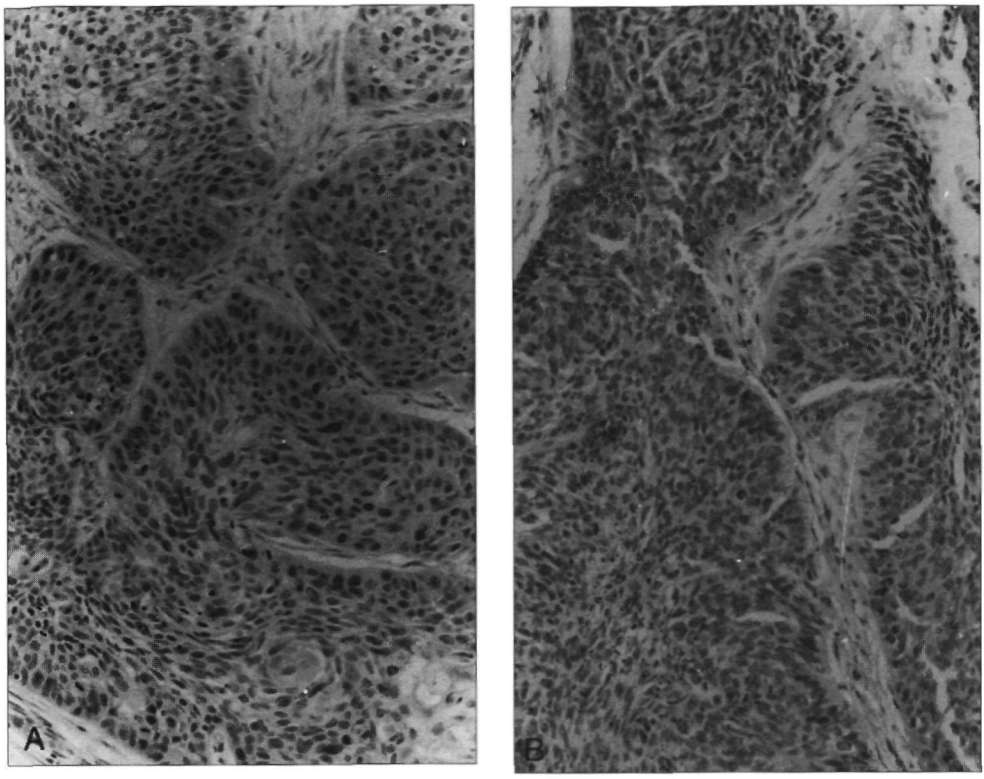

Figure 3.1. Micrographs of the original tumour A (A) and after the second passage in nude mice (B), showing comparable histologic features. (Haematoxylin-eosin; x 185).

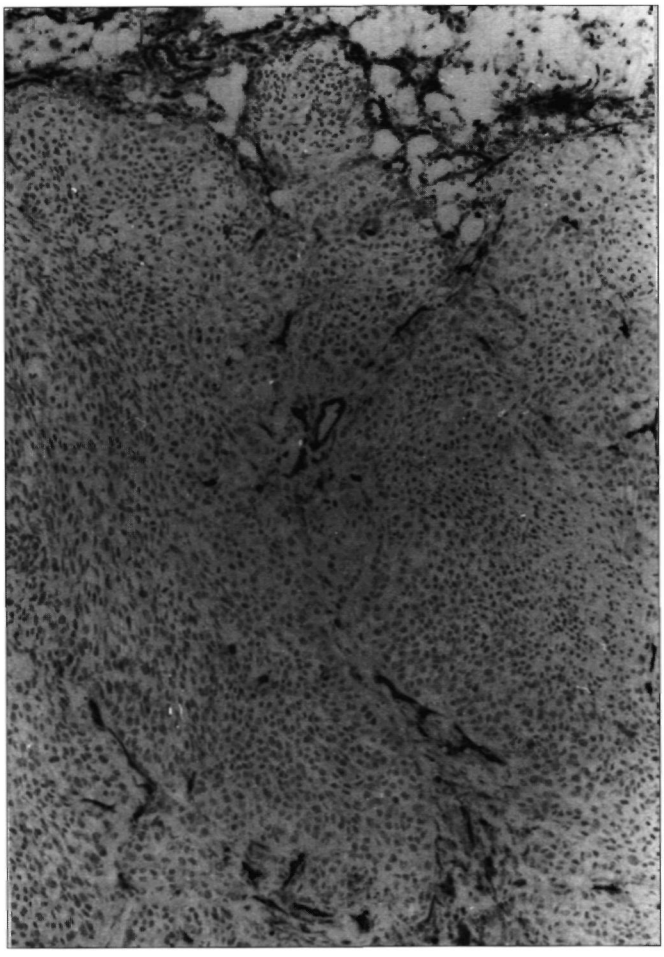

Figure 3.2. Immunohistochemical staining of xenograft, showing exclusive staining of vascular endothelium with the antibody MECA-20, specifically directed against mouse endothelium (x 90).

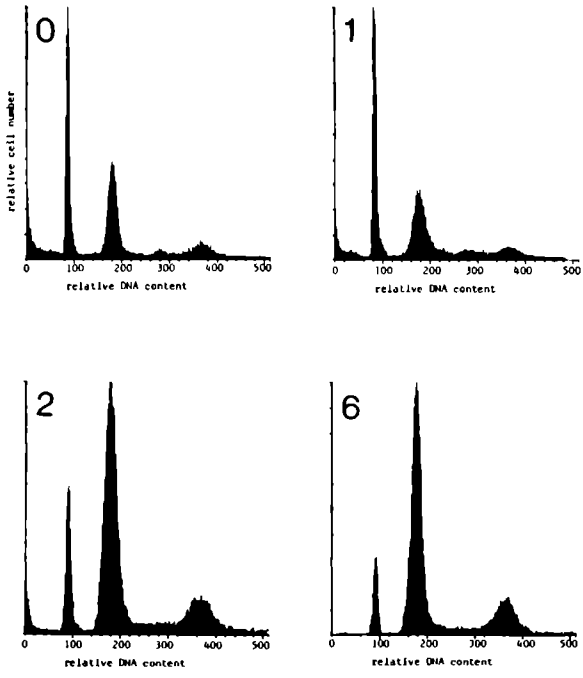

Figure 3.3. DNA histograms of tumour A. Flow cytometry was performed on the original tumour (0), the first (1), second (2), and sixth (6) passage in nude mice. The peak at channel 100 represents euploid cells.

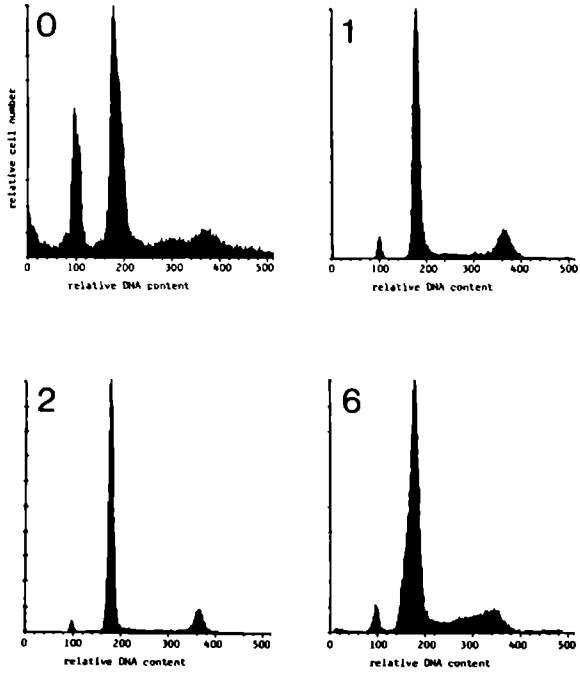

Figure 3.4. DNA histograms of tumour B. Flow cytometry was performed on the original tumour (0), the first (1), second (2), and sixth (6) passage in nude mice. The peak at channel 100 represents euploid cells.

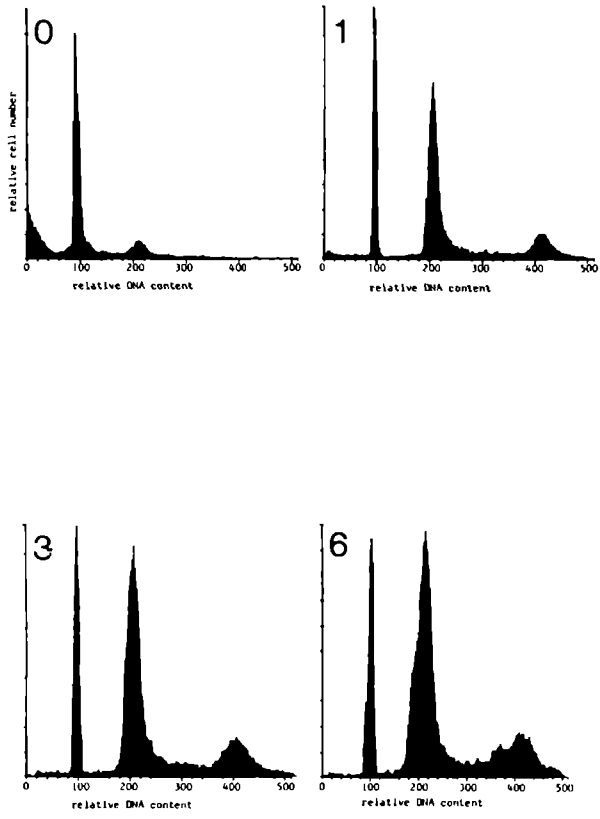

Figure 3.5. DNA histograms of tumour C. Flow cytometry was performed on the original tumour (0), the first (1), third (3) and sixth (6) passage in nude mice. The peak at channel 100 represents euploid cells.

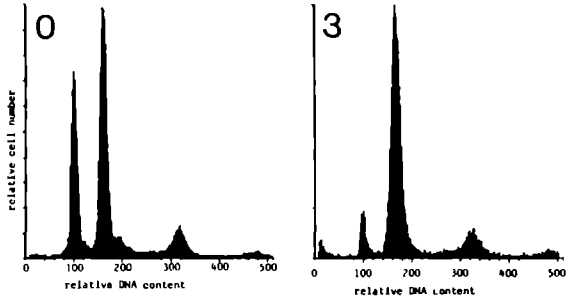

Figure 3.6. DNA histograms of tumour D. Flow cytometry was performed on the original tumour (0) and the third (3) passage in nude mice. The peak at channel 100 represents euploid cells.

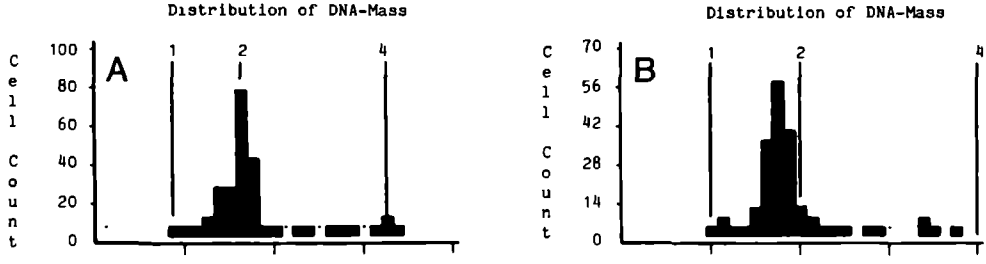

Figure 3.7. DNA image cytometric histograms of tumour tissue dissected from the xenografts of tumour A (12th passage, A) and tumour B (10th passage,B). The histograms show the exclusive presence of aneuploid cells (2). (1) indicates the position of normal cells, (4) the position of G2/M cells

the xenografts.
The histogram of primary tumour B demonstrated a small euploid and a large aneuploid DNA stem line with a DNA index of 1.8. At the first passage in nude mice, the proportion of euploid cells was severely reduced, while the aneuploid peak was virtually unaffected. This picture did not change in subsequent passages (Fig.3.4).
Flow cytometric analysis of primary tumour C showed a large proportion of euploid cells at channel 100 and a very small peak at about channel 200. During passaging in nude mice, the proportion of euploid cells and aneuploid cells changes in favour of the aneuploid portion (Fig.3.5). The DNA index of 2.2 was maintained during serial passaging.
The original tumour D had a large aneuploid peak and a smaller euploid peak. The DNA histogram of the xenograft at the third passage revealed a persisting high proportion of aneuploid cells, but a considerable decrease in the proportion of euploid cells. The DNA index (1.65) was found to be the same for the primary tumour and the xenograft (Fig.3.6).
It appeared that with DNA-flow cytometry no clear discrimination could be made between normal mouse cells and euploid human cells, because the DNA content of these species only slightly differs. For further identification of the euploid cells DNA image cytometry was applied on dissected tumour tissue, obtained from specimens embedded in paraffin. DNA image cytometric histograms of all four tumours studied revealed the exclusive presence of aneuploid cell populations as shown in Fig.3. 7 for tumours A and B. The DNA index of all four tumours did not differ significantly from that established with DNA flow cytometry.

5. Discussion

In this study four tumour lines of human squamous cell carcinoma of the head and neck region were successfully established in the nude mouse. It appeared that the initial lag-phase (the parameter for growth (10)) of all tumour lines decreased after the first transplantation while the take rate improved. In subsequent passages these parameters remained stable, but the differences between the individual tumours were maintained.
The xenografts retained the histomorphological features of the original tumours with respect to both type and grade.
These findings generally support the findings made in a previous study on xenografts of head and neck squamous cell carcinomas (10) and on xenografts of a large variety of other tumours (2-9).
DNA cytometry showed that all the primary tumours contained both euploid and aneuploid cells. During passaging in nude mice all the tumours retained their original DNA index, which is in line with the observations made in human ovarian carcinoma, osteosarcoma and renal cell carcinoma (23-25). Only incidentally some xenografted tumours have shown instability of the DNA content during longterm xenotransplantation (24, 25).
During passaging tumours A,B,C and D revealed a distinct decrease in the relative proportion of euploid cells, while the proportion of aneuploid cells increased.

A comparable dominance of aneuploid cells has been reported by Wennerberg (21) and van Haaften-Day et al. (38) after transplantation of tumours showing a bimodal DNA distribution with DNA flow cytometry. Wennerberg (21) reported the complete disappearance of the euploid tumour cell population from three mixed euploid and aneuploid head and neck squamous cell carcinomas after xenografting in nude mice. Comparable observations have been made by van Haaften-Day et al (38) with ovarian tumours. It was suggested from both studies that a selection of aneuploid cells had taken place. This was assumed to be due to a higher growth rate or better metabolic adaptability of the aneuploid tumour cells to culture conditions in nude mice.

This phenomenon deserves further consideration. Generally, little attention has been paid to the character of the euploid cell population in solid tumours with a bimodal DNA distribution. Although the diploid cells can represent an additional tumour clone, most authors assume that the euploid component is usually composed of normal host cells (23, 39-41). To differentiate between normal human host cells and euploid tumour cells routine DNA flow cytometry alone is inadequate and other cytological parameters are needed to solve this problem. After transplantation of tumours containing both euploid and aneuploid cell populations in nude mice, the characterization of the euploid cells is further complicated by the presence of ingrown stroma cells. Immunohistochemistry with the antibody MECA-20 clearly showed that these cells are of mouse origin.

Normal mouse cells have a DNA content close to that of human cells and because the CV ranged between 2 and 5.5% the mouse cells could not visualized as a separate population in the presented histograms. The present DNA image cytometry analysis on different xenografts, without significant contamination of stroma cells, convincingly demonstrates that all tested tumours contain only aneuploid tumour cells. Therefore the euploid peak in the DNA histograms of the xenografts can be concluded to represent normal mouse stroma cells. The observed variation in the relative proportion of mouse cells must be ascribed to the varying amount of stroma cells in the different specimens.

These observations advocate a careful analysis of the euploid cell population in order to decide on the presence of only benign host cells or benign and malignant euploid cells.

Although the number of tumours tested is limited, this study appears to confirm that in accordance with the observations made in other tumours both histological features and DNA content of head and neck squamous cell carcinoma remain stable during serial passaging in the nude mouse.

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CHAPTER IV.

CHEMOSENSITIVITY TESTING OF XENOGRAFTED SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK REGION.

This chapter has been submitted for publication:
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1. Abstract

Eight squamous cell carcinomas from the head and neck region were established as xenograft lines in nude mice and tested for their sensitivity to the antineoplastic drugs bleomycin and cisplatin. Tumour volume, histology, DNA flow cytometry and mitotic activity were used as parameters.
One out of the 8 tumours appeared to be highly sensitive to bleomycin, while three other tumours were sensitive to both bleomycin and cisplatin. These observations are in good correlation with the reported data in patients.
All chemosensitive tumours showed regrowth after the cytotoxic drug treatment had been completed. No change was seen in the chemosensitivity or other features of the regrown tumours not even after repeated exposure to the drugs.
Comparison of the tumour volume with the other parameters applied, indicated that the tumour volume of squamous cell carcinomas was not always a reliable parameter for testing chemosensitivity because of the considerable contribution of keratin to the tumour volume. It is concluded that additional parameters such as histological examination, DNA flow cytometry or mitotic activity, are necessary in order to draw reliable conclusions on xenografts with a large avital component. In addition, DNA flow cytometry has proved to be very useful for the rapid screening of drug sensitivity.

2. Introduction

Tests to predict the drug sensitivity of tumours outside the patient include both in vitro culture techniques and xenografts in immunologically incompetent laboratory animals.
Good correlations for some of the in vitro assays have been reported between in vitro chemosensitivity and the patient's response (1). However, the disadvantage of this approach is that the tumour cells are cultured in an artificial environment and lack the influence of the host's metabolism, while the pharmacokinetic properties of the drugs are not reflected in the therapeutic outcome. Although metabolism and pharmacokinetics will differ in some respects in xenogeneic hosts, the results of drug testing of tumours grown in these hosts may be assumed to have more clinical relevance.
From all xenogeneic hosts the athymic nude mouse has received the most interest since its introduction by Rygaard and Povlsen (2) as a model for studying the behaviour of human tumours and for testing antineoplastic drugs (3).
Xenografts of a large variety of tumours can be succesfully grown in this animal throughout many generations, without any significant changes in the tumour characteristics (4-8).
The main drawback of this animal model is that a direct comparison between the chemosensitivity of a xenograft and the patient is limited, because of the long delay between establishing the xenografts and drug testing. In addition, the activity of methotrexate, shown to be very effective on head and neck squamous cell carcinoma, was less pronounced on xenografts than could be expected from clinical studies (9).

Despite these limitations, this model has proved to be valid in retrospective studies and as a predictive system for testing new cytotoxic drugs (5,9-17).
The criteria for evaluating the effects of anticancer drugs on xenografts have not yet been standardized. The evaluation is mainly based on changes in the tumour volume (5), tumour growth delay (18) or the life span of the animal (19). Cell cycle disturbances have been used incidentally as parameters (20-22), but histological observations have only rarely been performed (23).
We evaluated the effect of cisplatin and bleomycin therapy on xenografts of 8 squamous cell carcinomas from the head and neck region using the parameters: tumour volume, histology, DNA cytometry and labeling index. These two antineoplastic drugs have been shown to be effective for the treatment of these carcinomas (24).

3. Materials and Methods

3. 1. Tumours

We studied 8 heterotransplanted squamous cell carcinomas from the head and neck region, which had been transplanted through a varying number of passages (Table 4.I.). The procedure for serial transplantation in nude mice has been described in detail elsewhere (7). Briefly, the tumour grafts were removed under ether anaesthesia and cut into pieces of about 1-2 mm^3. These tissue fragments were implanted subcutaneously into the flanks of about 30 nude mice (weight about 20 g). Tumour growth was measured using a Vernier caliper at weekly intervals or more frequently in the case of rapidly growing tumours. The tumour volume was calculated as 0.5 x the product of the three dimensions and was plotted on a linear scale (25).

3. 2. Chemotherapy

To study the effect of cytotoxic drugs on tumour growth, tumour bearing animals were randomized into control and treatment groups, consisting of about 10 animals each. The treatment groups received either bleomycin or cisplatin at a maximum tolerated dose, while the control groups received a saline injection. Bleomycin (Lundbeck) was dissolved in water and injected subcutaneously into the back of the mice. The animals were treated with a total dose of 100 mg bleomycin, administered at a dose rate of 25 mg every two days. Cisplatin (cis-dichlorodiamino-platinum) (Bristol Meyers) was dissolved in water immediately before intraperitoneal injection. The animals received a total dose of 12 mg/kg body weight, administered as a single dose of 3 mg/kg every two days. Treatment was initiated about 3-5 weeks after transplantation when the tumours had reached a size, which varied between 100 and 300 mm^3.

3. 3. DNA flow cytometry

For flow cytometry (FCM), tumour tissue was minced with a scalpel and washed twice in Hank's balanced salt solution. The tissue was subsequently suspended in Eagle's minimal essential medium (MEM) containing collagenase (2mg/ml, type

Table 4.I. Chemosensitivity of xenografts of head and neck squamous cell carcinomas.

Tumour	Origin	Stage	Histology	Initial lag phase 1)	DNA index 2)	Chemosensitivity Bleomyc.	Cispl
1. HN-4	Piriform sinus	T2N1M0	Mod. diff., keratinizing	1-4 wks.	1.6	+	+
2. HN-6	Tongue	T2N0M0	Mod. to well diff., keratinizing	1-4 wks.	1.8	+	-
3. HN-9	Supraglottic	T3N0M0	Mod. to well diff., keratinizing	1-2 wks	1.0	-	-
4. HN-2	Epiglottic	T2N1M0	Mod. diff., keratinizing	2-4 wks.	1.6	-	-
5. HN-2M	Lymph node metast. of HN-2	T2N1M0	Mod. diff., keratinizing	3-5 wks.	2.4	-	-
6. HN-13	Flour of the mouth	T2N0M0	Mod. to well diff., keratinizing	1-2 wks.	2.0	+	+
7. HN-15	Trigonum retromol.	T2N1M0	Mod. to well diff., keratinizing	1-3 wks.	2.2	+	+
8. HN-14	Epiglottis	T1N1M0	Mod. to well diff., keratinizing	2-3 wks.	1.8	-	-

1) Time needed to obtain a mean diameter of 4 mm during serial passaging.
2) DNA-index is defined as the ratio between the channel number of the aneuploid peak and that of the diploid peak of the same sample.

II, 132 U/mg: Cooper biomedical) and hyaluronidase (0.5 mg/ml, type I, 370 U/mg; Sigma) and incubated under agitation for 1 hour at 37°C. After the tissue suspension had settled, the supernatant was discarded and the tissue was incubated in Hank's balanced salt solution, containing trypsin (0.25 mg/ml) and dithioerythritol (3 mg/ml) overnight at 4°C. The suspension was spun down at 200 g and the pellet was resuspended in 4 ml MEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Europe) and 100 µl RNase (2.5 mg/ml) and incubated for 30 minutes at 37°C under mild agitation. After centrifugation of the supernatant at 200 g, the cells were fixed in 70% ethanol.
Aliquots of 10^6 cells/ml were stained in phosphate buffered saline (PBS) containing 1 mM EDTA, propidium iodide (40 µg/ml; Calbiochem) and 100 µl/ml RNase (1 mg/ml) for 10 minutes at 37°C and subsequently filtered through a 50 µm nylon mesh. The DNA content was measured using flow cytometry (Ortho Diagn, MA; system 30 H). Human lymphocytes stained with propidium iodide were used for optical alignment of the FCM apparatus. The amplifier gain setting was adjusted until the (diploid) lymphocytes fell in channel 100 on a 512 channel scale. Generally, 25,000 cells were measured per histogram. The coefficient of variation (CV) of the standard was approximately 2% and varied between 2 and 5.5% for the tumour cells. DNA flow cytometry was carried out on the xenografts during the pretreatment period and repeated 1, 2, 3 and 4 weeks after the start of the treatment with cytotoxic drugs.
The modal DNA content, expressed as a DNA index, was defined as the ratio between the channel number of the aneuploid peak and that of the euploid peak of the same sample.

3. 4. DNA image cytometry
Formalin-fixed, paraffin embedded tumours were used for image cytometry. This method has been described in detail elsewhere (26). Briefly, 50 µm sections were dewaxed with xylene and rehydrated. Guided by adjacent haematoxylin-eosin stained sections, the tumour tissue was dissected and incubated in PBS, containing 0.1% protease for 1 hour at 37°C. The isolated nuclei were washed twice in PBS and counted with a coulter counter. Approximately 30,000 nuclei were spun down and resuspended in 200 µl fetal bovine serum. A monolayer smear was prepared on a glass slide, using a special centrifugation bucket. The slide was air dried and fixed in a mixture containing methanol, formaldehyde and acetic acid (85:10:5 v/v) for 1 hour. The smears were stained with pararosanilin and the DNA content of 200 nuclei was measured using the CAS system. Normal human cells were used as a control.

3. 5. Histology and autoradiography
For the histological studies, the entire xenografts were fixed in phosphate buffered (0.1M; pH 7.4) formaldehyde (4 %) and embedded in paraffin. Sections (7 µm) were stained with haematoxylin-eosin.
To evaluate the effect of the cytotoxic drugs on DNA synthesis, animals in both the control and treatment groups received an intraperitoneal injection of ^{3}H-thymidine (1µCi/ gram body weight; S.A. 2Ci/mM, Radiochemical centre, Amersham,

England). All the animals were injected at 10.00 a.m. to avoid diurnal variations . The animals were killed by decapitation one hour later and processed as described above. Sections (7 μm) were mounted on gelatinized slides and coated with Ilford G5 emulsion using the dipping technique (27). After an exposure time of 4 weeks the sections were developed , fixed and stained with haematoxylin-eosin.
A total of 1,000 tumour cells were counted at 4 different sites in each xenograft. The labeling index was expressed as the number of labeled cells per 100 cells counted.

4. Results

4. 1. Untreated tumours

4. 1. 1. Growth behaviour

The xenografted tumours grew as circumscribed, often lobulated tumours, surrounded by a fibrous capsule. In two tumours, infiltration of tumour cells into the capsule was observed. No distant metastases were found. All the xenografts displayed an increasing amount of necrosis and/or accumulation of keratin or parakeratotic lamellae in the central part of the lobules within a few weeks after transplantation. During serial passaging all the tumours showed a stable growth pattern, i.e. the time needed for the tumour to reach a mean diameter of about 4mm defined as the initial lag-phase (7) (Table 4.I). The histological features of the primary tumours appeared to have been retained.
The mitotic activity of tumours HN-4, HN-6 and HN-9 was determined using ^{3}H-thymidine. No great differences between the labeling indices of the tested tumours were observed. The labeling index was found to be rather stable throughout the observation period between 3 and 14 weeks after transplantation (Table 4.II).

4. 1. 2. DNA cytometry

DNA flow cytometry of the various tumour lines showed an euploid and an aneuploid peak in all tumours except for tumour HN-9 which displayed only a single euploid peak. The DNA indices are listed in Table 4.I.
DNA image cytometry of the dissected parts of the xenografts revealed that the euploid peak of the tumours with a bimodal DNA distribution represented normal mouse cells, as illustrated by tumour HN-13 in Fig. 4.1. The tumour which showed only an euploid peak with DNA flow cytometry (HN-9), was found to contain only euploid tumour cells.

4. 2. Effect of cytotoxic drugs

The effects of bleomycin and cisplatin on xenografted squamous cell carcinoma of the head and neck region in nude mice are summarized in Table 4.I. Three tumours (HN-4, HN-13 and HN-15) were found to be sensitive to both drugs, while one tumour (HN-6) was only sensitive to bleomycin. The remaining four tumours (HN-9, HN-2 and its lymph node metastasis HN-2M and HN-14) were not sensitive to either of the drugs.

Table 4.II. Incorporation of 3H-thymidine (labeling index; mean ± SD) in xenografts at various times after start of drug administration.

Tumour	Drug	start treatment	10 days after start	4 weeks after start	9 weeks after start
HN-4	Contr	28.6 ± 3.8	25.5 ± 4.0		26.4 ± 1.2
HN-4	Cispl		0.0 ± 0.0	27.0 ± 5.1	24.5 ± 4.7
HN-6	Contr	21.7 ± 2.7		23.9 ± 3.2	20.0 ± 2.8
HN-6	Cispl		30.0 ± 2.6	21.3 ± 2.8	16.0 ± 2.4
HN-6	Bleom		0.0 ± 0.0	22.4 ± 4.6	20.9 ± 2.0
HN-6*	Contr	19.7 ± 0.6	19.9 ± 2.2	28.7 ± 4.2	23.9 ± 3.4
HN-6*	Bleom		0.0 ± 0.0		20.3 ± 3.4
HN-9	Contr	24.9 ± 3.3	20.1 ± 0.7	23.6 ± 3.9	19.0 ± 3.2
HN-9	Cispl		21.4 ± 8.7	22.0 ± 1.1	
HN-9	Bleom		29.9 ± 4.3	24.0 ± 0.4	21.2 ± 4.8

* Tumours raised from cells of tumour HN-6, which regrew after first treatment with bleomycin.

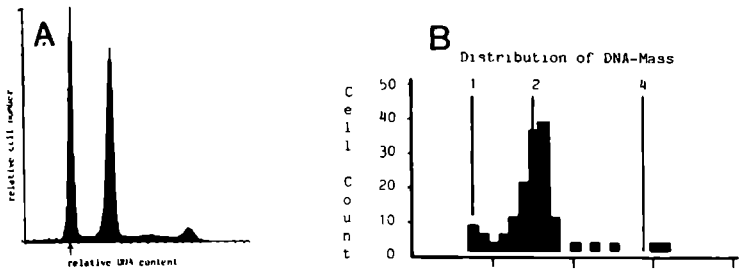


Figure 4.1. DNA cytometry of xenografts of tumour HN-13 (4th passage). A. DNA flow cytometry (arrow indicates euploid cells at channel 100); B. DNA image cytometric histogram of dissected tumour cells, showing the exclusive presence of aneuploid tumour cells (2). (1) indicates the position of euploid cells; (4) indicates position of tetraploid cells.

4. 2. 1. Growth curves

A sharp decrease, starting during the first week and leading to nearly complete regression of the tumour was observed in the growth curves of tumours HN-4 and HN-13 within 4 to 5 weeks after the start of cisplatin treatment. This was followed by gradual regrowth of the tumours. This is shown for tumour HN-4 in figure 4.2.A. The same patterns were also observed for these tumours after exposure to bleomycin.

The growth curves of tumours HN-6 and HN-15 flattened out after exposure to bleomycin, but 4 to 5 weeks after the completion of drug treatment, the tumour volume gradually increased as shown for tumour HN-6 in figure 4.2.C. The growth curves of tumour HN-15 showed a comparable behaviour after cisplatin treatment, but this drug did not have any effect on the growth curves of tumour HN-6.

The growth curves of tumours HN-9, HN-2, HN-2M and HN-14 did not show any change after treatment with cisplatin or bleomycin in comparison with the control group of the same tumours. There were no changes in the response of drug sensitivity testing during the various passages up to the 12th passage. In addition, the xenografts established from tumour cells (HN-4 and HN-6), which regrew after one, two and three courses of treatment with cytotoxic drugs, did not show any change in their drug sensitivity or growth pattern, as shown in Fig. 4.2.B and D.

4. 2. 2. Histology

Histological examination of the different tumours at the start of drug exposure showed circumscribed, occasionally lobulated tumours, surrounded by a fibrous capsule. The tumours consisted of a varying amount of vital tumour cells and dead material, which was composed of cellular debris, dyskeratosis or keratin lamellae. The composition of this material differed between the various tumour lines. Examples are given in Figs. 4.3 A and 4.4 A.

Histological examination of the sensitive tumours demonstrated vacuolisation and cytolysis of the tumour cells, which lead to profound degeneration of the major part of the tumour cells between 1 and 2 weeks after the start of drug exposure. At two weeks, the tumours were mainly composed of dead material. In tumours HN-4 and HN-13 this material consisted of cellular debris, dyskeratosis and fluid surrounded by a fibrous capsule of varying thickness (Fig. 4.3 B). The stroma showed severe infiltration by inflammatory cells (Figs. 4.3 B, C; 4.4 B, C). Scattered tumour cells could be found in and on the inner side of the fibrous capsule (Figs. 4.3 C; 4.4 C). In tumours HN-6 and HN-15 the dead material mainly consisted of keratin lamellae (Fig. 4.4 B). Between two and three weeks onwards, the number of tumour cells started to increase, initially mainly in the peripheral part of the tumours (Figs. 4.3 D; 4.5 A, B). This finally resulted in complete regrowth of the tumours. In tumours HN-4 and HN-13 tumour regrowth was associated with an increased proliferation of fibrous tissue (Fig. 4.3 D).

4. 2. 3. Labeling index.

The injection of ^{3}H-thymidine after drug administration, revealed a fast decrease in the number of labeled cells to zero between one and two weeks in tumours HN-4 and HN-6 after treatment with cisplatin and bleomycin, respectively. Four weeks

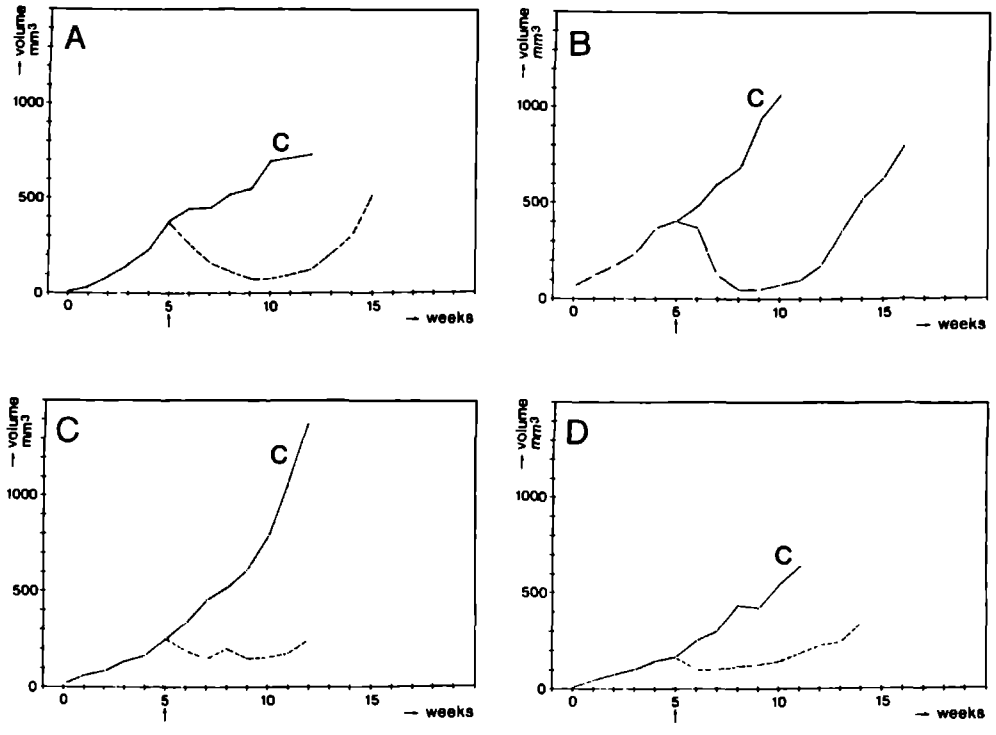
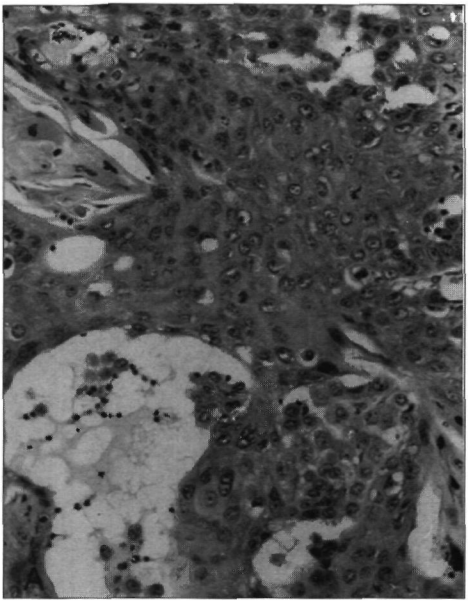
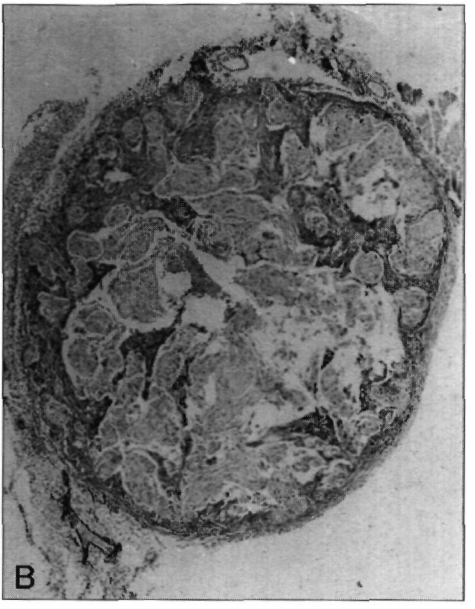


Figure 4.2. Typical examples of the effects of cisplatin administration on growth curves of xenografts of tumour HN-4 (A) and of bleomycin on HN-6 (C), showing two different types of curves. Growth curves B and D represent the effect of drug administration on xenografts raised from the tumour cell population regrown after three successive treatments. Arrow indicates the start of drug injection. c: untreated xenografts.

Figure 4.3. Micrographs of xenografts of tumour HN-4, before (A) and 2 weeks (B) and 4 weeks (D) after the start of cisplatin administration. C. higher magnification of B, showing scattered tumour cells. Regrowth of tumour is visible in D. (Haematoxylin-eosin; A,C,D: x 185; B: x 20).

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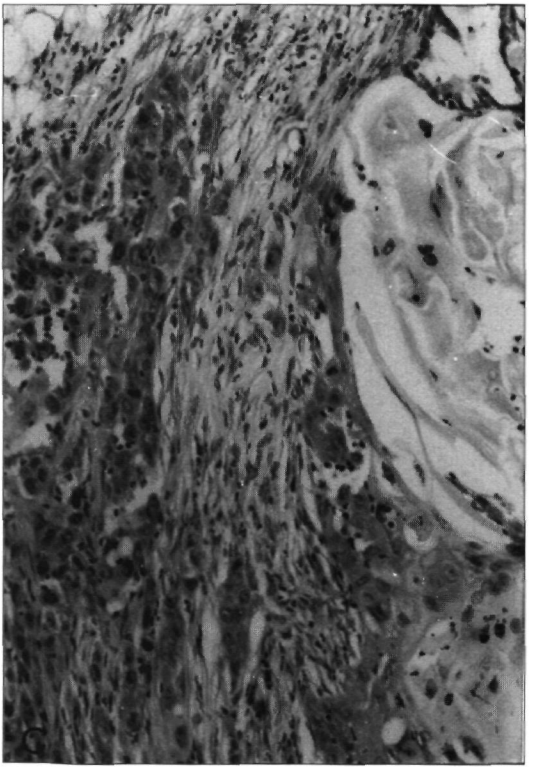

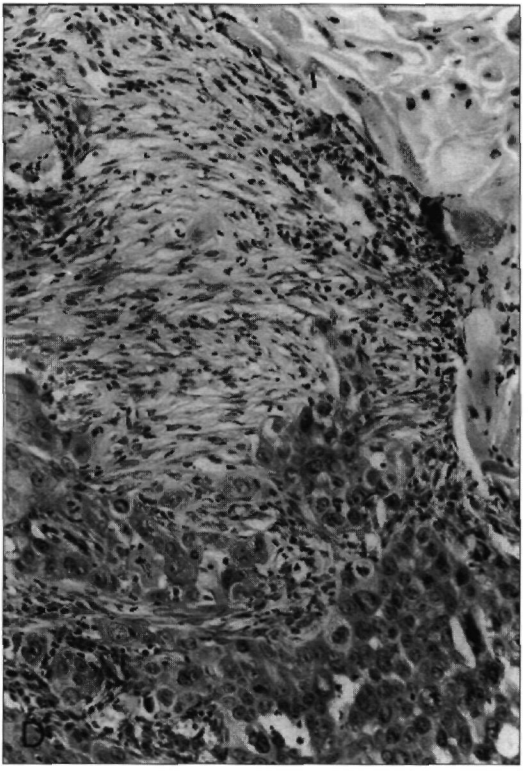
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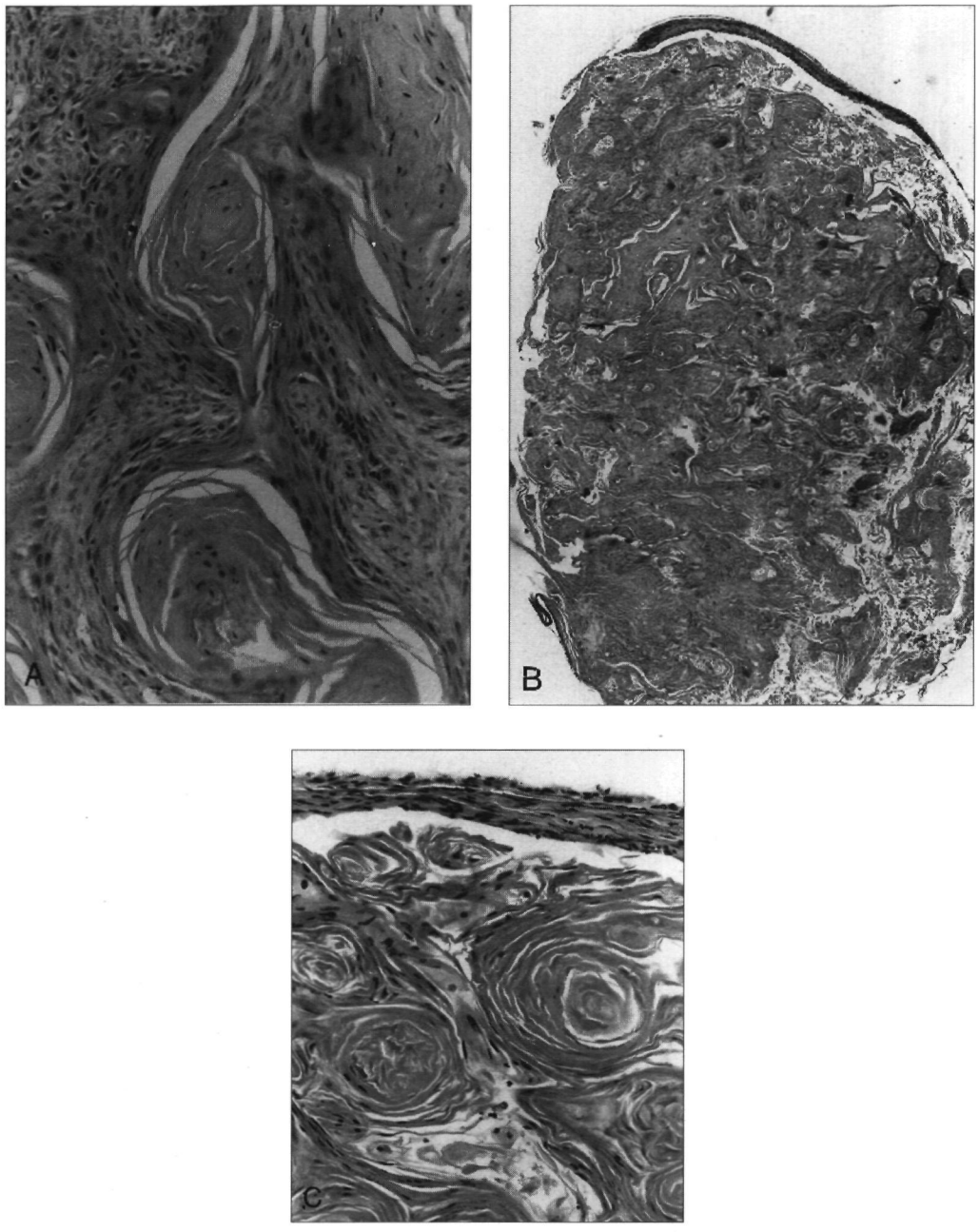

Figure 4.4. Micrographs of xenografts of tumour HN-6, before (A) and 2 weeks (B) after the start of bleomycin administration. C. higher magnification of B, showing accumulated keratin lamellae and fibrous capsule. (Haematoxylin-eosin; A,C: x 185; B: x 20).

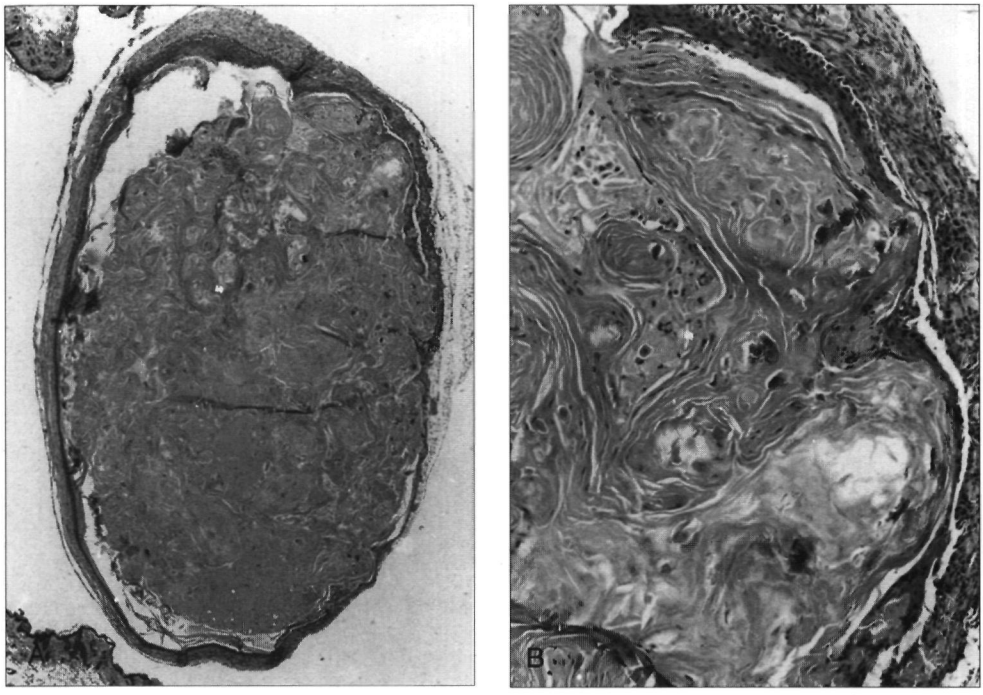

Figure 4.5. Survey (A) and higher magnification (B) of xenograft of tumour HN-6, four weeks after the start of bleomycin administration, showing regrowth of the tumour on the inner side of the fibrous capsule. (Haematoxylin-eosin; A: x 15; B: x 100).

after the start of drug administration, the labeling-index of the regrown tumours had returned to the pretreatment level (Table 4.II). Comparable observations were made when a xenograft of tumour HN-6, raised from a regrown cell population of cells after the first treatment with bleomycin, was treated with bleomycin. The labeling indices of the tumours, which according to their growth curves and histology did not respond to cisplatin (HN-6 and HN-9) or bleomycin (HN-9) did not significantly differ from the control values (Table 4.II).

4. 2. 4. DNA flow cytometry

The DNA histograms of the xenografts, which according to their growth curves and histology were found sensitive to cisplatin or bleomycin (HN-4, HN-6, HN-13 and HN-15) showed a sharp decrease in the aneuploid peak and the G2/M peak one week after the start of drug administration, while there was a relative increase in the proportion of the mouse stroma cells. Depending on the tumour line, the DNA histograms regained their pretreatment profiles between two and four weeks after the start of drug exposure (Figs. 4.6, for example tumours HN-13 and HN-15). A similar course was observed for the tumours raised from the regrown tumours which were treated for a second and a third time.

No changes were seen in the DNA histograms of the tumours which were found to be insensitive to treatment by bleomycin (HN-2, HN-2M, HN-9 and HN-14) or cisplatin (HN-2, HN-2M, HN-6, HN-9 and HN-14) (Figs. 4.7, for example tumours HN-9 and HN-14).

5. Discussion

Human tumour xenografts grown in nude athymic mice form a valuable model for studying tumour behaviour and testing antineoplastic drugs.

In the present study, 8 human squamous cell carcinomas from the head and neck region, established as xenograft lines in nude mice, were tested for their sensitivity to cisplatin and bleomycin using different parameters. The results showed that 1 out of the 8 xenografts was highly and selectively sensitive to bleomycin (HN-6). Three tumours (HN-4, HN-13 and HN-15) were sensitive to both drugs and the remaining four tumours (HN-9, HN-2, HN-2M and HN-14) were not sensitive to either of the drugs. These conclusions were drawn on the basis of histological examination, the growth curves and DNA flow cytometry. This was further substantiated by the study of the incorporation of ^{3}H-thymidine in three tumours. No correlation was found between the chemosensitivity and the degree of differentiation, which is in agreement with the observations made in tumours from other origins (28,29).

The present observations are also in line with clinical response with these drugs. Partial or complete regression has been observed in the same order of magnitude (30,31) while differential sensitivity for either cisplatin or bleomycin has been established.

The differential sensitivity is in agreement with the scarce available data on chemosensitivity testing on xenograft lines of head and neck squamous cell carcinomas, performed by Sneeuwloper et al. (32) and Azar et al. (33). Remarkably,

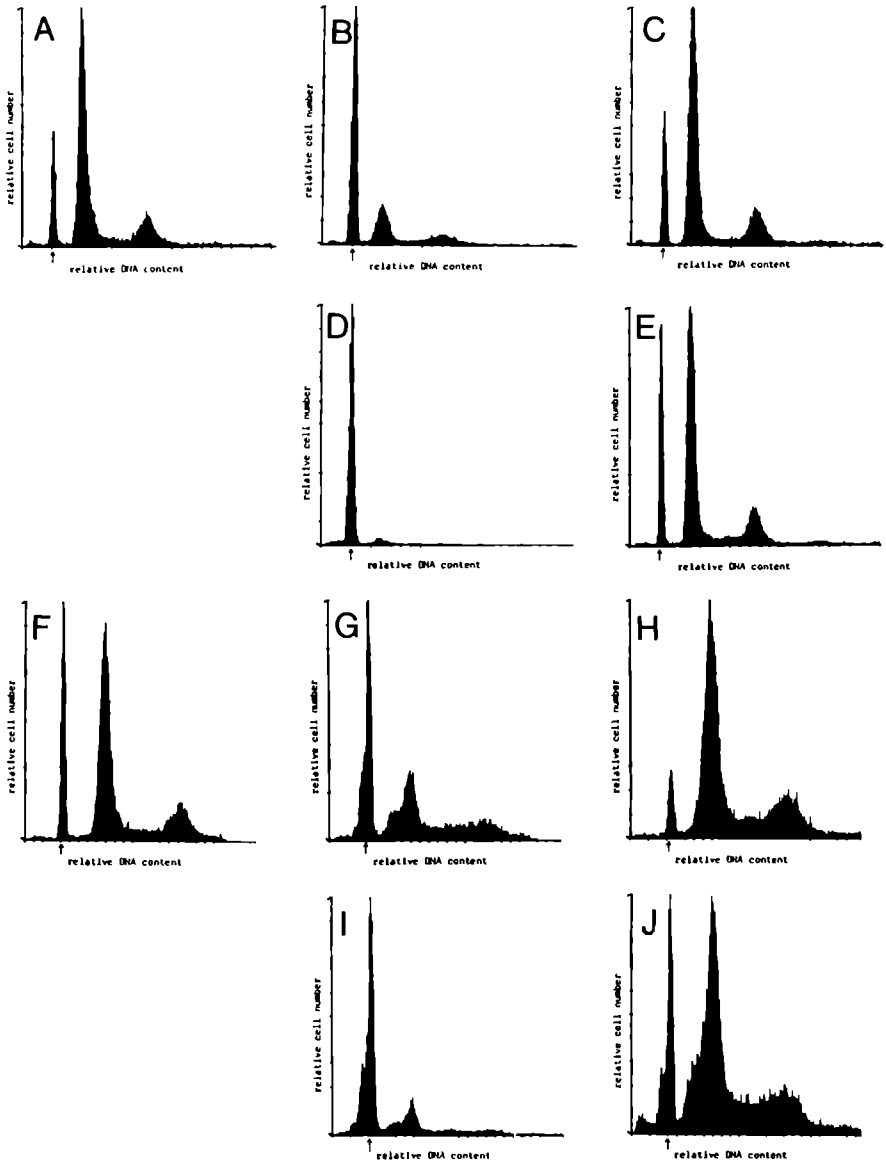

Figure 4.6. DNA histograms of xenografts of tumours HN-13 and HN-15 at various times after the start of drug administration. The tumours are sensitive to both cisplatin and bleomycin.
Tumour HN-13: A. control; B. cisplatin 1 week; C. cisplatin 3 weeks; D. bleomycin 1 week; E. bleomycin 2 weeks.
Tumour HN-15: F. control; G. cisplatin 1 week; H. cisplatin 2 weeks; I. bleomycin 1 week; J. bleomycin 2 weeks.
(arrows indicate euploid cells at channel 100)

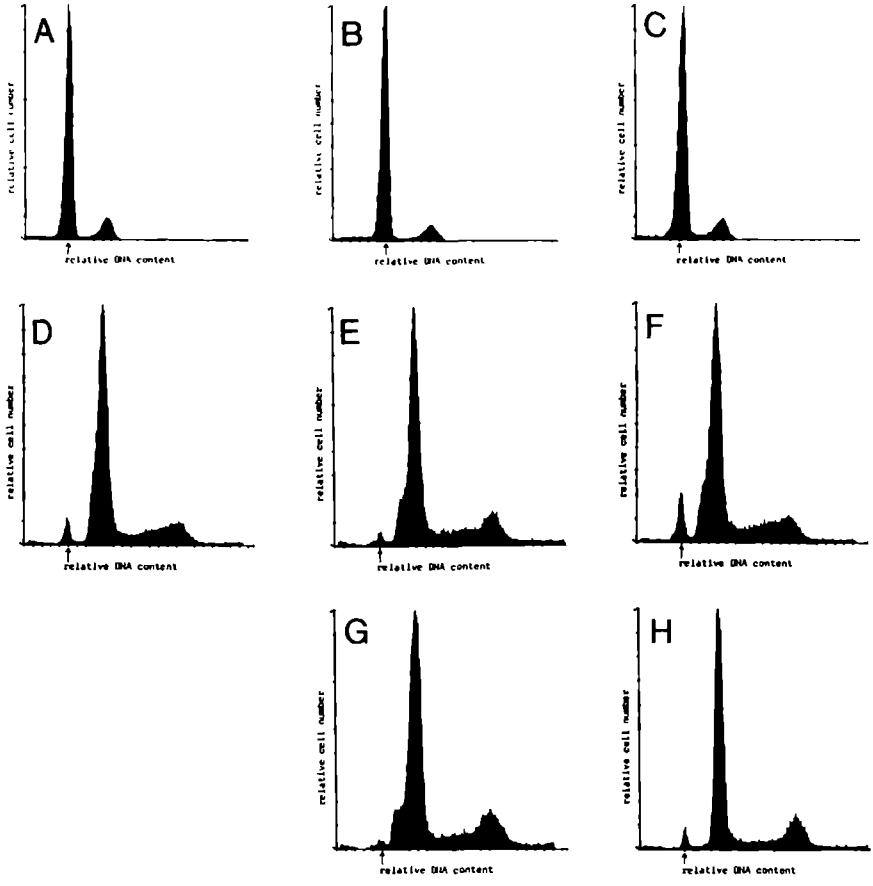

Figure 4.7. DNA-histograms of xenografts of tumours HN-9 and HN-14 at various times after the start of drug administration. These tumours are insensitive to both cisplatin and bleomycin. Tumour HN-9: A. control; B. cisplatin 1 week; C. bleomycin 1 week. Tumour HN-14: D. control; E. cisplatin 1 week; F. cisplatin 2 weeks; G. bleomycin 1 week; H. bleomycin 2 weeks.
(arrows indicate euploid cells at channel 100).

Braakhuis et al. (9) reported sensitivity to both drugs in 6 out of 7 xenograft lines tested, using the tumour volume as an evaluation parameter. This is a high sensitivity rate in comparison with the present data and the clinical observations reported by Taylor (34). However, there was wide variation in the sensitivity of the different lines. When they omitted the tumours with only a minimal change in the tumour volume, a close correlation with the clinical findings could be found.

In the present study, two types of growth curves could be distinguished after cytotoxic drug treatment. One type showed a sharp decrease in the tumour volume and can be considered to represent a strong response. The other type, showing a flattening of the curves, which can be considered to depict a moderate response or growth retardation, without any significant regression of the tumour volume. However, in both cases histopathological examination showed the nearly complete disappearance of tumour cells between one and two weeks after the start of drug treatment. A comparable observation was made with DNA flow cytometry. The only difference observed was that in the tumours with the most marked response the central part of the xenografts contained a varying amount of fluid, cellular debris and dyskeratosis. The moderately responding tumours, however, showed in addition to cellular debris, the presence of a large mass of keratin lamellae surrounded by a fibrous capsule. These findings indicate that flattening of the growth curve is related to the accumulation of keratin lamellae, which are more resistant to desintegration and resorption and prevent a further decrease in the tumour volume after the disappearance of the tumour cells.

Although the volume is generally considered to be a useful parameter for the evaluation of drug sensitivity of tumour xenografts, the present findings indicate that drug-induced changes of the tumour volume are not always a true reflection of the response to drug treatment. If the sensitivity of the tumour is only moderate or low or if it contains both sensitive and insensitive populations of tumour cells, the question arises as to whether the tumour size is a reliable parameter for the response to treatment if a large amount of dead material is present. Therefore, the final evaluation of a tumour's response to cytotoxic drugs in xenografts has to be made in conjunction with histopathological examination.

The DNA histograms of chemosensitive tumours showed distinct changes in the population of tumour cells within one week after the start of drug administration. These rapid changes in the DNA histograms of chemosensitive tumours, indicate that DNA flow cytometry may be very useful for the rapid screening of drug effects.

All the sensitive tumours showed regrowth, usually within 2-3 weeks after treatment, although the maximum tolerated drug dose has been applied. The first signs of regrowth were detected by DNA flow cytometry and histological examination after 2-3 weeks, followed by a gradual increase in the tumour volume.

The tumour cells which regrew were most likely the original tumour cells, which had escaped drug treatment. This can be concluded on the basis of the DNA indices of the tumours after regrowth which were similar to those of the control tumours and also on the labeling indices of the regrown tumours HN-4 and HN-6, which were not significantly different from the pretreatment levels. Moreover, these parameters were found to be the same after retransplantation of the regrown

cell population. Although tumour resistance has often been reported during patient treatment after previous cytotoxic drug therapy, to our knowledge, this phenomenon has been reported incidentally in xenograft lines of human tumours. Regrowth of effectively treated human tumour xenografts has been reported by Kyriazis et al. (23) in a comparable study. These authors suggested that this regrowth was caused by resistant tumour cell clones, although no further proof was given. Using a more convincing approach, Mattern et al. (35) studied the development of drug resistance in xenografts of an epidermoid lung carcinoma. They observed a great variation in the time course of the development of resistance against the various drugs applied. Resistance to vincristin and actinomycin D was observed after one single treatment and was suggested to be due to the presence of a pre-existing subpopulation of cells, which were resistant to these drugs in the original tumour. Conversely, resistance to cisplatin did not become apparent until after the 4th treatment and it slowly progressed after subsequent treatments. This gradual development of tumour resistance against cisplatin has also been found in other xenografts (36,37). These observations might explain the absence of any change in the therapeutic response during 3 successive treatments in the present study

Summarizing, this study shows that tumour volume, histology, ^{3}H-thymidine incorporation and DNA cytometry can be used as parameters for evaluating drug response. Growth curves alone appear not to be a fully reliable parameter for the final evaluation of drug sensitivity, especially for tumours containing a substantial amount of dead material with low or moderate sensitivity. In these cases, additional parameters are needed.

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CHAPTER V.

A DEVICE FOR THE PRODUCTION OF FRESH TUMOUR SLICES FOR CYTOTOXIC DRUG TESTING

This chapter has been published in: Tumor Biol 10: 324-329, 1989 by Elprana D, Schwachöfer J and Kuijpers W.

1. Abstract

The potential suitability of thin slices of fresh tumour tissue for the in vitro testing of antitumour drugs was studied.
With a simple device, sets of tumour slices of equal thickness were produced rapidly with minimal cellular damage.
The sections showed a reproducible stable incorporation of ^{3}H-thymidine for incubation periods of more than 24 hours.
Preliminary data on the effect of cytotoxic drugs on sections of xenografts of human squamous cell carcinoma, revealed a distinct correlation between their in vitro effects on ^{3}H-thymidine incorporation and their in vitro sensitivity in nude mice.

2. Introduction

Over the past decade, studies on the development of in vitro assays for predicting the in vivo drug sensitivity of solid malignant tumours has been mainly focussed on the use of single cell suspensions both in short and long-term cultures (1-7). Although good correlation has been reported between some types of tumours and in vivo conditions, there is still no consensus that any of these tests are suitable for routine clinical use.
One of the major limitations of these techniques is the preparation of representative suspensions of single viable cells. It has become clear from several studies that the disaggregation procedure used, either mechanical, enzymatic, chemical or a combined procedure, has great influence on the vitality, number and type of cells isolated (8, 9).
In order to overcome these problems, the use of tumour fragments or slices seems to be a good alternative. However, this technique has only been applied incidentally for cytotoxic drug testing (10), presumably because of the problems encountered in obtaining comparable tissue fragments with equal proportions of representative viable tumour cells, preferably from the same tumour region.
The major advantage of this approach lies in the preservation of cell to cell and cell to matrix interactions. Moreover, serious cell damage caused by the disruption of intercellular connections which is especially pronounced in some epithelial tumours, can be prevented. In this report we describe a simple device for the rapid production of thin slices from fresh tumour tissue with a comparable number of viable tumour cells and their suitability for cytotoxic drug testing.

3. Materials and Methods

The construction of the device for the production of thin tumour slices is shown in Fig. 5.1. The device consists of four razor blades assembled in a stainless steel holder. The razor blades are approximated by stainless steel blades of 0.4 mm and bolted together.

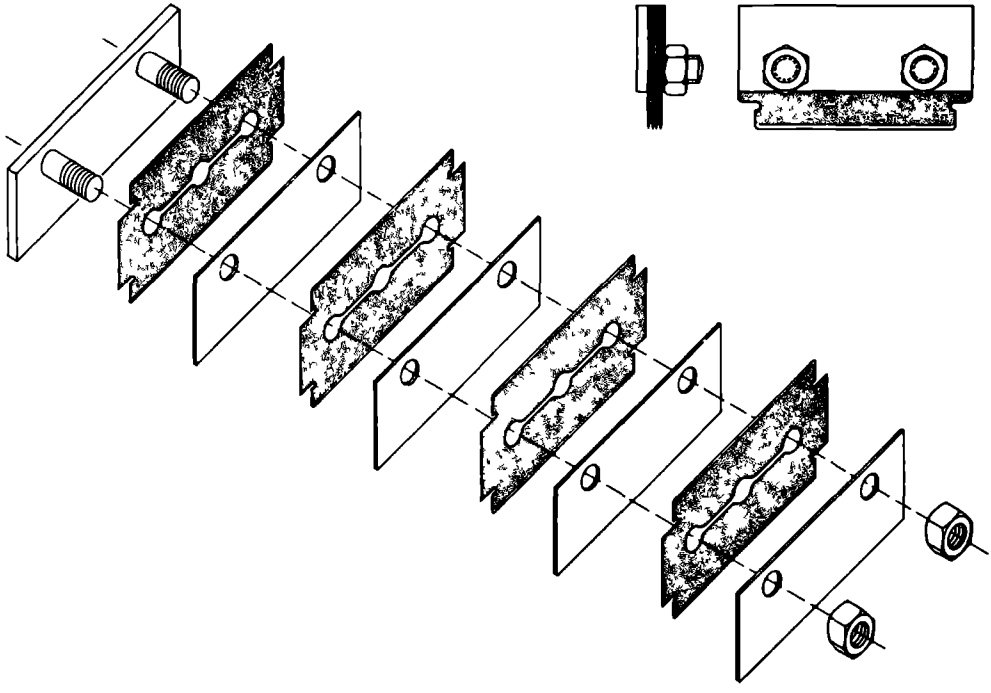

Fig. 5. 1. Slicing device, consisting of 4 razor blades separated by stainless steel blades of 0.4 mm

In this study, use was made of tumour xenografts obtained from established lines of human head and neck squamous cell carcinomas in nude mice (11).
For slicing, part of the tumour, trimmed to an appropriate size, was submerged in tissue culture medium (MEM) in a Petri dish to avoid drying out. The blades were advanced through the tumour tissue using a single gentle stroke. In this way, sets of three tumour slices could be obtained.
After loosening the bolts, the slices could easily be removed from between the blades with a fine camel hair brush and deposited in the tissue culture medium. The slices were subsequently transferred to small flasks containing 2 ml incubation fluid. Incubation was performed in RPMI 1640 with 10% foetal bovine serum supplemented with 2 mM glutamin/ml, 100 IU penicillin/ml and 100 μg streptomycin/ml (Gibco Ltd., Paisly, Scotland) for various periods.
For cytotoxic drug testing, either cisplatin or bleomycin were added to a final concentration of 10 and 5 μg/ml, respectively.
During the last hour of incubation, ^{3}H-thymidine (S.A. 2 Ci/mM, Radiochemical centre, Amersham, England) was added to the culture medium to a final activity of 1μCi/ml. As hyperbaric oxygenation has been shown to favour the uptake of ^{3}H-thymidine in the deeper parts of the tumour slices (12) incubation was carried out under hyperbaric conditions (1.8 atm. O_2).
After incubation the slices were washed in an isotonic solution of unlabelled thymidine for 3 hours at 4°C to remove any free ^{3}H-thymidine and subsequently dissolved in Luma Solve (Lumac Systems AG, Basel, Switzerland). A LKB 81000 Liquid Scintillation Counter was used to measure the ^{3}H-thymidine incorporation.
To determine the site of ^{3}H-thymidine incorporation, the slices were fixed with neutralized formaldehyde (4%) after incubation and embedded in paraffin. Sections of 7 μm were coated with Ilford K.5 emulsion and developed after an exposure time of 4 weeks. Staining was performed with haematoxylin-eosin.

4. Results

Sets of three slices of tumour tissue could be cut in rapid succession with the device. Without much practice, 5 sets could be obtained within 15 minutes. The wet weights of several sets of slices, obtained from a block of tumour tissue trimmed to an appropriate size, are summarized in Table 5.I. These data demonstrate that the variation in weight between the different slices from each set are rather small.
The autoradiograph in Fig. 5.2 shows the specific incorporation of ^{3}H-thymidine into the nuclei of the tumour cells througout the whole slice.
For measuring the inter-slice variation of ^{3}H-thymidine incorporation, sets of three slices from the same tumour were used. The slices were incubated separately. The data obtained for one of the tumours, presented in Table 5.II, show good comparability between the amount of ^{3}H-thymidine incorporated into each set of slices.
An example of the incorporation of ^{3}H-thymidine after various incubation periods is shown in Fig. 5.3. Incorporation, measured as cpm/slice, was expressed as a percentage of the value measured after one hour, which was set at 100%. It

Table 5.I. Wet weight of individual slices of 4 sets obtained from one block of tumour material.

Set no.	Weight (mg)	Average (± SD)
1a	15.3	
b	16.7	
c	16.4	16.1 ± 0.7
2a	10.2	
b	12.5	
c	9.8	10.8 ±1.5
3a	13.4	
b	12.8	
c	14.3	13.5 ± 0.8
4a	14.8	
b	14.2	
c	13.7	14.2 ± 0.6

Table 5.II. ^{3}H-thymidine incorporation in 4 sets of tumour slices, measured in cpm/slice. Different slices from each set were incubated separately to determine inter-assay variation.

Set no.	cpm/slice	Average (± SD)
1a	7,354	
b	7,668	
c	7,475	7,499 ± 158
2a	8,819	
b	8,676	
c	8,693	8,748 ± 101
3a	9,117	
b	8,977	
c	9,228	9,107 ± 126
4a	9,879	
b	9,798	
c	9,683	9,787 ± 98

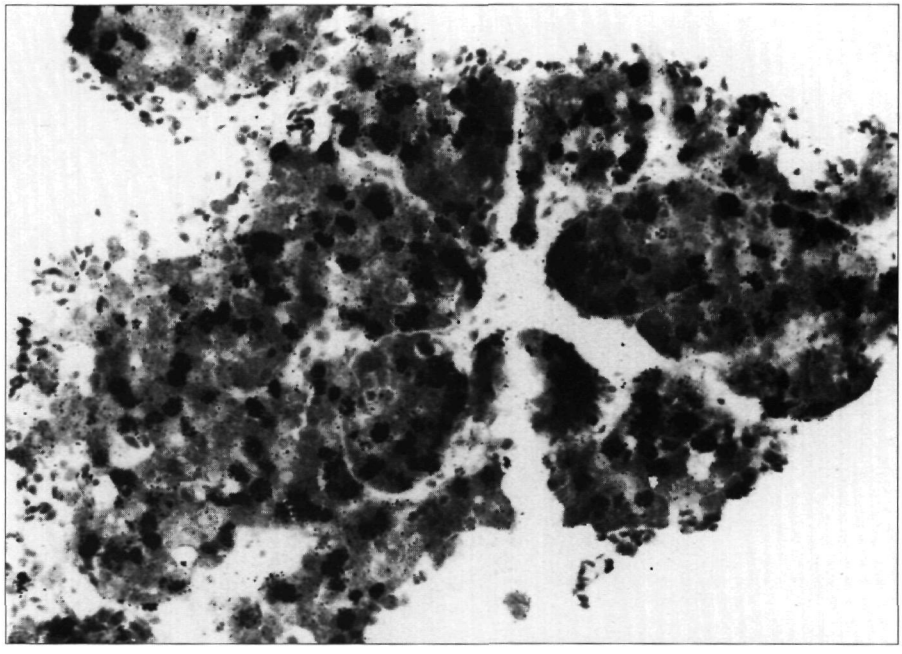

Fig. 5. 2. Micro-autoradiograph of tumour slice incubated for 5 hours at 1.8 atm O_2. ^{3}H-thymidine was added during the last hour of incubation. Note the specific incorporation of ^{3}H-thymidine in the nuclei of the tumour cells, distributed throughout the whole section. (Haematoxylin-eosin, x 270).

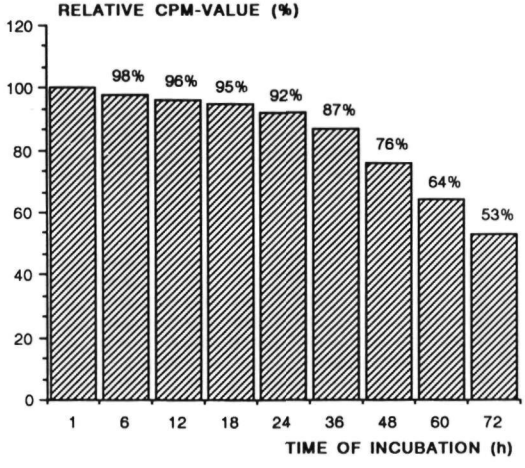

Fig. 5. 3. ^{3}H-thymidine incorporation in tissue slices of tumour xenograft during increasing incubation periods; cpm-values are expressed as a percentage of the value measured after 1 hour of incubation (11,748 cpm). ^{3}H-thymidine was added during the final hour of incubation.

appeared that incorporation in time only slightly decreased during the first 24 hours. The radioactivity subsequently decreased to about half of the original value during the next 48 hours.
A preliminary study on 4 xenografts from different tumour lines, to investigate the feasibility of this method for cytotoxic drug testing was performed. The data revealed only a substantial decrease in the incorporation of ^{3}H-thymidine in the tumours found to be sensitive to the same drugs in vivo in nude mice, as shown in Table 5.III (13).

Table 5.III. The effect of cisplatin and bleomycin on the incorporation of ^{3}H-thymidine in slices of xenografts from 3 different squamous cell carcinomas of the head and neck and 1 lymph node metastasis (HN-2M lymph node metastasis of HN-2) after an incubation period of 24 hours.(Mean ± SD of 3 slices). ^{3}H-thymidine incorporation in control sections was set at 100%. ^{3}H-thymidine was added during the final hour of incubation.
** A comparable effect was found in vivo in nude mice with these drugs (ref. 13)*

Tumour	Cisplatin*	Bleomycin*
HN-2M	108.75 ± 27.50%	93.75 ± 11.25%
HN-2	85.0 ± 6.25%	92.50 ± 12.50%
HN-4	45.0 ± 21.25%	38.75 ± 17.50%
HN-6	81.25 ± 25.0 %	37.50 ± 17.50%

5. Discussion

The present study was undertaken to investigate the feasibility of using thin tumour slices to test cytotoxic drugs in vitro. Through the preservation of tissue integrity, this approach has the great advantage that artefacts, associated with the use of single-cell suspensions, are avoided.
It was established that with the simple device presented, sets of comparable slices from fresh tumour tissue could be produced very rapidly without special skill. The tumour slices prepared in this way showed minimal cellular damage or metabolic disturbances, as can be concluded from the reproducible incorporation of ^{3}H-thymidine, which remained stable throughout an incubation period of at least 24 hours.
Especially the production of sets of serial slices which can be considered mutually comparable with respect to their cell population and ^{3}H-thymidine incorporation, makes this method very suitable for cytotoxic drug testing. In this way, sections treated with drugs can be compared with an adjacent control section.
The close correlation established between the effects of some antitumour drugs on slices of xenografted human squamous cell carcinoma and the in vivo response in

nude mice, justify the broader application of this approach on tumour specimens directly obtained from patients.

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CHAPTER VI.

CYTOTOXIC DRUG SENSITIVITY OF SQUAMOUS CELL CARCINOMAS AS PREDICTED BY AN IN VITRO TESTING MODEL

This chapter has been published in: Anticancer Res 9: 1098-1094, 1989 by Elprana D, Schwachöfer J, Kuijpers W, van den Broek P and Wagener DJTh.

1. Abstract

Two in vitro techniques for testing the chemosensitivity of squamous cell carcinomas from the head and neck were compared.
Single-cell suspensions and tumour slices obtained from human xenografts were used. The sensitivity to cisplatin and bleomycin was quantified using the incorporation of ^{3}H- thymidine as a parameter. The in vitro assays were compared with in vivo tests in mice.
No reliable data could be obtained with cell suspensions of these tumours due to a rapid decrease in cell viability during incubation while tumour slices revealed a stable control level of ^{3}H-thymidine incorporation for up to 30 hours.
In 6 out of 7 cases close agreement was found between the effect of the cytotoxic drug on the tissue slices and on the same tumours in nude mice.

2. Introduction

Clinical experience has shown that treatment modalities which are effective for one tumour type are not necessarily effective for another, even tumours of the same type or histology exhibit wide variability in their response to cytotoxic agents. The development of predictive tests which can determine the sensitivity or resistance of malignant tumours to antineoplastic agents prior to therapy, has brought about a variety of testing systems (1). Both in vivo and in vitro assays have emerged, which apply different parameters to quantify tumour response to drugs.
Each test has both advantages and disadvantages in terms of sensitivity, accuracy and financial feasibility.
Although the subcutaneous implantation of human tumuor xenografts in athymic or immunosuppressed mice is fairly reliable with regard to its accuracy for predicting the sensitivity to certain drugs (2,3,4), it will always be unsuitable for individual patient testing due to its expensive and time-consuming character.
An alternative in vivo method: the subrenal capsule assay, has been introduced by Bogden et al. (5). This method involves the implantation of solid pieces of tumour tissue under the renal capsule. Drug sensitivity can be tested within 6 days and the predictive data of this assay have produced promising clinical correlations (6), with an accuracy comparable with conventional subcutaneous implantation (7).
From recent studies it has become clear that it is necessary to make histologic assessments of this model with regard to its suitability for predicting the effect of cytotoxic drugs (7, 8).
When employing in vitro predictive tests on human tumour cells, a number of general technical problems must be overcome, such as cell death, contamination, low growth rate and low plating efficiency. In particular the single-cell suspensions which employ the incorporation of radioactive labeled nucleotide precursors (9) and the stem cell assay introduced by Hamburger and Salmon (10), which determines the reproductive capability of tumour cells, have proved to be potential tools for quantifying the drug sensitivity of human tumours.
Although the human tumour stemcell assay is subject to a variety of artefacts, in

recent years there has been growing interest in its applicability in prospective clinical trials due to its potential capacity to predict drug resistance in certain tumour types (11,12). The routine selection of anticancer drugs is still premature, but being able to predict the resistance in 84-95% of the cases and drug sensitivity in 40-70% of the cases, seems promising (13). However, only a limited number of tumour types can be used effectively in the stemcell assay and only a subset of these, including breast, colorectal, kidney, lung and ovarian tumours and melanomas, give rise to evaluable assays (14).
To our knowledge, the stemcell assay is the only in vitro test to have been applied for testing the chemosensitivity of human head and neck tumours. In their study, Johns and Mills (15) showed that 49% of 73 squamous cell carcinomas demonstrated clonal growth. They concluded that a high cloning efficiency correlates with a poor prognosis. This was contradicted by Mattox et al. (16) who failed to find such a correlation. The high rate of contamination, low growth rate and low plating efficiency were the main problems encountered. This led to the conclusion that a clonogenic assay does not contribute to the management of patients with cancer of the head and neck. In a recent study, it appeared that the different techniques used for cell dispersion of this type of tumour have a great deal of influence on the quality of the cell suspension (17). This may be an underlying cause for the low plating efficiency.
The present study deals with the in vitro testing of the chemosensitivity of human head and neck squamous cell carcinoma, using both cell suspensions and tissue slices. The results obtained in vitro were compared with the in vivo effects in nude mice.

3. Materials and Methods

3. 1. Tumours

The tumours used in this study were xenografts of human squamous cell carcinomas from the head and neck region, grown subcutaneously in nude mice (Balb/c, nu/nu). The origin and histological features of these tumours are shown in Table 6. I. The tumours were transplanted through 10-15 passages and were found to preserve their histological features (18). Apart from these xenografts, one original well-differentiated squamous cell carcinoma from the tongue (T4M3M0) was used.

3. 2. Testing of antitumour drugs

3. 2. 1. Cell suspensions

Tumour tissue, obtained from xenografts in mice, was minced with razor blades under sterile conditions. The fragments were then incubated for 16 hours at 4°C in PBS containing trypsin (0.25 mg/ml) and dithioerythritol (3 mg/ml) as described by Bijman et al. (17). The suspensions obtained were subsequently heated to 37°C and agitated until complete disaggregation of the tissue had occurred. They were then passed through a sterile nylon filter (Ø 70 μm) and collected in Eagle's Minimal Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS) (All tissue-culture materials were obtained from Gibco B.V., Breda, The

Table 6.I. Origin and histology of xenografts and their chemosensitivity to cisplatin and bleomycin determined in vivo (nude mice) and in vitro (tumour slices)

Tumour	Origin	Stage	Histology	CHEMOSENSITIVITY in vivo Cispl.	in vivo Bleom.	in vitro Cispl.	in vitro Bleom.
1. HN-2	Epiglottic	T2N1M0	Mod. diff. keratinizing squamous cell carcinoma	-	-	-	-
2. HN-2M	Lymph node metast. of HN-2	T2N1M0	Mod. diff. keratinizing squamous cell carcinoma	-	-	-	-
3. HN-4	Pirifom sinus	T2N1M0	Mod. diff. keratinizing squamous cell carcinoma	+	+	+	+
4. HN-6	Tongue	T1N0M0	Mod. to well diff. keratinizing squamous cell carcinoma	-	+	-	+
5. HN-13	Flour of the mouth	T2N0M0	Mod. to well diff. keratinizing squamous cell carcinoma	+	+	+	+
6. HN-14	Epiglottis	T1N1M0	Mod. to well diff. keratinizing squamous cell carcinoma	-	-	+	-
7. HN-15	Trigonum retromol.	T2N1M0	Mod. to well diff. keratinizing squamous cell carcinoma	+	+	+	+

Netherlands). After washing the cells twice for 5 min. in fresh MEM + 10% FBS, the suspensions were diluted to a final concentration of 1.0 x 10^6 cells/ml and divided over three incubation flasks, each containing 15 ml suspension. Cell viability, determined by trypan blue exclusion, varied between 65 and 80%. Cisplatin (Platinol; Bristol Myers, Madrid, Spain) was added to the first flask and bleomycin (Lundbeck, Copenhagen, Denmark) was added to the second. The final concentrations were 10 and 5 μg/ml, respectively. The third flask served as a control.

The flasks were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. At regular intervals, 4 ml samples were taken from the flasks and divided over four test-tubes, 1 ml each, to which ^{3}H-thymidine (S. A. 4 Ci/mmol; Radiochemical Centre, Amersham, England) was added. The final ^{3}H-thymidine concentration was 1 μCi/ml. After incubation for a further hour, ice-cold unlabeled thymidine was added to the test-tubes. The cells were then washed and collected on a filter (Schleicher & Schüll, no. 9) and subsequently dissolved in Luma Solve (Lumac Systems AG, Basel, Switzerland). The level of radioactivity incorporated in the samples was measured in a LKB 81000 Liquid Scintillation Counter.

3. 2. 2. Tumour slices

The tumour xenograft was extirpated under sterile conditions and transferred into cold MEM to a laminar flow cabinet for further preparation. Three adjacent slices of tumour tissue were obtained by using a device consisting of four razor blades mounted in a holder at a distance of 0.40 mm (19). For slicing, part of the tumour, trimmed to an appropriate size, was submerged in tissue culture medium in a Petri dish to avoid drying out. The blades were advanced through the tumour using a single gentle stroke. After loosening the bolts, the slices could easily be removed from the blades with a fine camel brush and deposited in the tissue culture medium. The slices were subsequently transferred to small flasks containing 2 ml incubation fluid. One slice was incubated in a test-tube containing 1.8 ml MEM plus 0.2 ml saline to serve as a control. The other two were placed in test-tubes containing MEM to which either bleomycin or cisplatin dissolved in saline was added. The final concentrations were 10 μg cisplatin/ml and 5 μg bleomycin/ml, respectively. For each assay three sets of three slices were incubated at 37°C for varying periods of time. Incubation was performed under hyperbaric conditions (1.8 atm. O_2), because hyperbaric oxygenation has been shown to enhance the uptake of ^{3}H-thymidine in the deeper areas of tumour slices (20). During the last hour of incubation ^{3}H- thymidine was added to the test-tubes to a final concentration of 1 μCi/ml. The slices were then washed in an isotonic solution of unlabelled thymidine for 3 hours at 4°C and subsequently dissolved in Luma Solve (Lumac Systems AG, Basel, Switzerland). Radioactivity was measured as described above. The cpm values of the ^{3}H-thymidine incorporation measured in the control slices were set at 100%. The effects of the drugs were expressed as percentages of the control.

3. 2. 3. Autoradiography

To establish the specificity of ^{3}H-thymidine incorporation, tumour slices were fixed in neutralized formaldehyde (4%) after incubation and processed for

embedding in paraffin wax. Sections (7 μm) were coated with Ilford K5 emulsion and developed after an exposure time of 4 weeks. Staining was performed with haemotoxylin-eosin.

3. 3. Testing in mude mice

For in vivo chemosensitivity testing, tumour-bearing nude mice were treated at a tumour size varying between 100 and 300 mm^3. Bleomycin (dissolved in water) was administered subcutaneously at a dose of 25 mg/kg body weight at 48-hour time intervals, to a final dose of 100 mg/kg body weight.
Cisplatin (dissolved in water) was given intraperitoneally at a dose of 3 mg/kg body weight at 48 hour-time intervals, to a final dose of 12 mg/kg body weight.
The drug effects on the size of the tumours were measured with a Vernier caliper at two days intervals. Mice injected with water according to the same schedule, served as controls.

4. Results

4. 1. Chemosensitivity testing in cell suspensions

The incorporation of ^{3}H-thymidine in single cell suspensions of 6 tumours is illustrated in Fig. 6.1. All suspensions show a steep decrease in ^{3}H-thymidine incorporation during an incubation period of 4 hours in both control samples and samples to which cytotoxic agents were added. This observation makes it impossible to draw reliable conclusions regarding the effects of the drugs tested.

4. 2. Chemosensitivity testing in tumour slices

A typical example of the uptake of ^{3}H-thymidine in tumour slices throughout an incubation period of 86 hours is shown in Table 6.II. These data demonstrate that the isotope incorporation remained virtually unchanged for 30 hours. Thereafter a gradual decrease occurred resulting in about 50% of the initial value at 86 hours.
Autoradiographs of the tumour slices revealed a specific incorporation of ^{3}H-thymidine in the nuclei of the tumour cells (Fig. 6.2).
To determine the inter-slice variation of ^{3}H-thymidine incorporation, sets of three slices of each tumour were used. The slices were incubated separately. The data obtained for tumour HN-13 demonstrated that there was good comparability between the different sets of slices (Table 6.III). Comparable results were obtained for other xenografts and it was established that small tumours gave the best reproducibility. This might be related to the amount of keratin and cell debris present.
The results of the sensitivity tests on cisplatin and bleomycin in all the tumours tested are summarized in Fig. 6.3. Based on the interslice variation in ^{3}H-thymidine incorporation, it was decided to conclude that there had been an inhibitory effect when the decrease in isotope incorporation was at least 30%. Initially, isotope incorporation was measured at 2, 5 and 24 hours, but because the inhibitory effect was most pronounced after 24 hours and no relevant information was obtained from the 2 measuring points during the first 5 hours, chemosensitivity testing was limited to one point at 4 hours in the later tests.
The data presented in Fig. 6.3 show that both the primary tumour (HN-2) and the

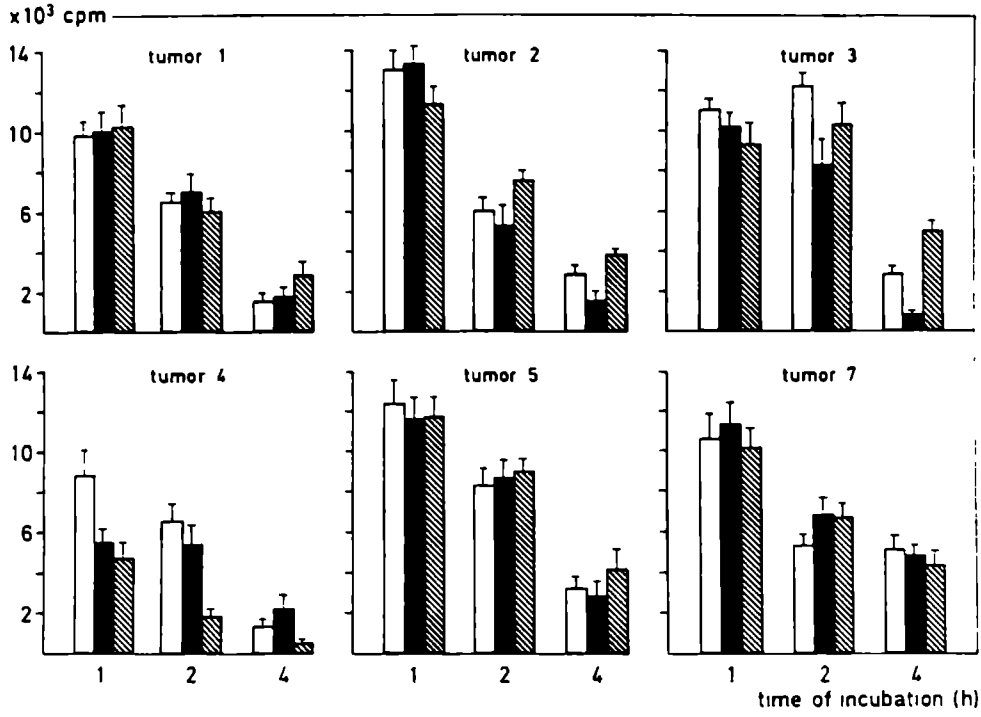

Figure 6.1. Incorporation of ^{3}H-thymidine in single-cell suspensions from six squamous cell carcinoma xenografts (see Table 6. I) incubated for various periods with cisplatin (black bars) and bleomycin (dashed bars). There is a rapid decrease in ^{3}H-thymidine incorporation in both treated and control samples (open bars) with progression of time. ^{3}H-thymidine was added during the final hour of incubation. Each bar represents the mean ± SD of four samples.

Table 6.II. ^{3}H-thymidine incorporation (cpm/slice) in tumour slices of tumour (HN-15) after various incubation periods. ^{3}H-thymidine was added during the last hour of incubation.

Time of incubation (h)	cpm/slice	(% of initial value)
2	12,342	(100%)
6	11,848	(96%)
18	11,725	(95%)
30	11,478	(93%)
48	10,738	(87%)
60	8,516	(69%)
72	7,899	(64%)
86	6,048	(49%)

Table 6.III. ^{3}H-thymidine incorporation in 5 sets of tumour slices of tumour (HN-13) incubated for two hours. ^{3}H-thymidine was added during the last hour of incubation. Each figure represents the mean (cpm/slice) of a set of three slices.

Mean	S.D.	S.D. as percentage of mean
10,563	995	9.4
9,952	739	7.4
7,500	278	3.7
7,223	328	4.5
12,810	1,300	10.1

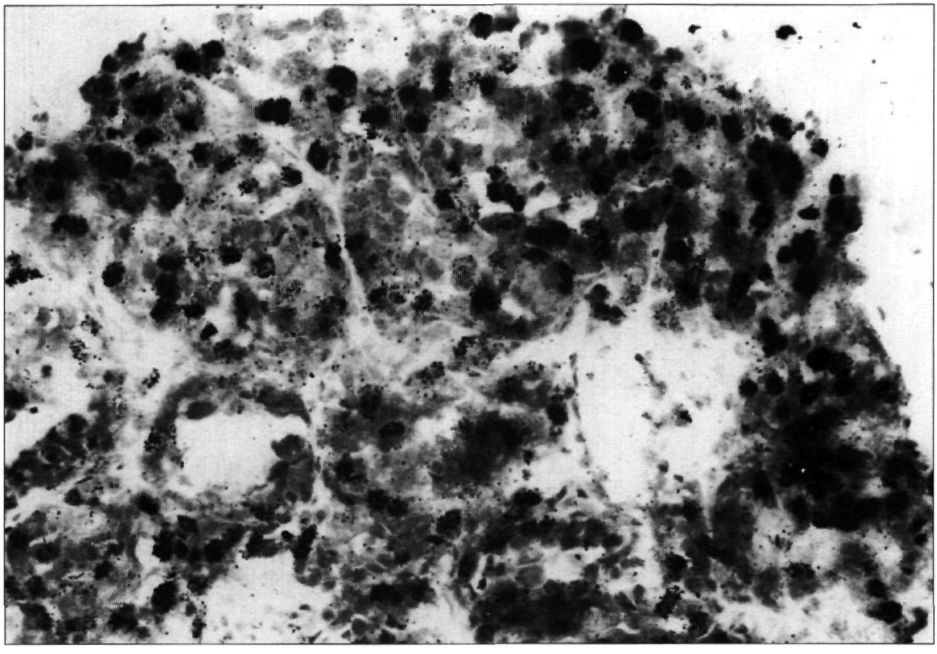

Figure 6.2. Micro-autoradiograph of a tumour slice incubated for 5 hours. in MEM + saline at 1.8 atm. O_2. ^{3}H-thymidine was added during the final hour of incubation. Note the labelling of tumour cell nuclei throughout the whole section (haematoxylin-eosin, x270).

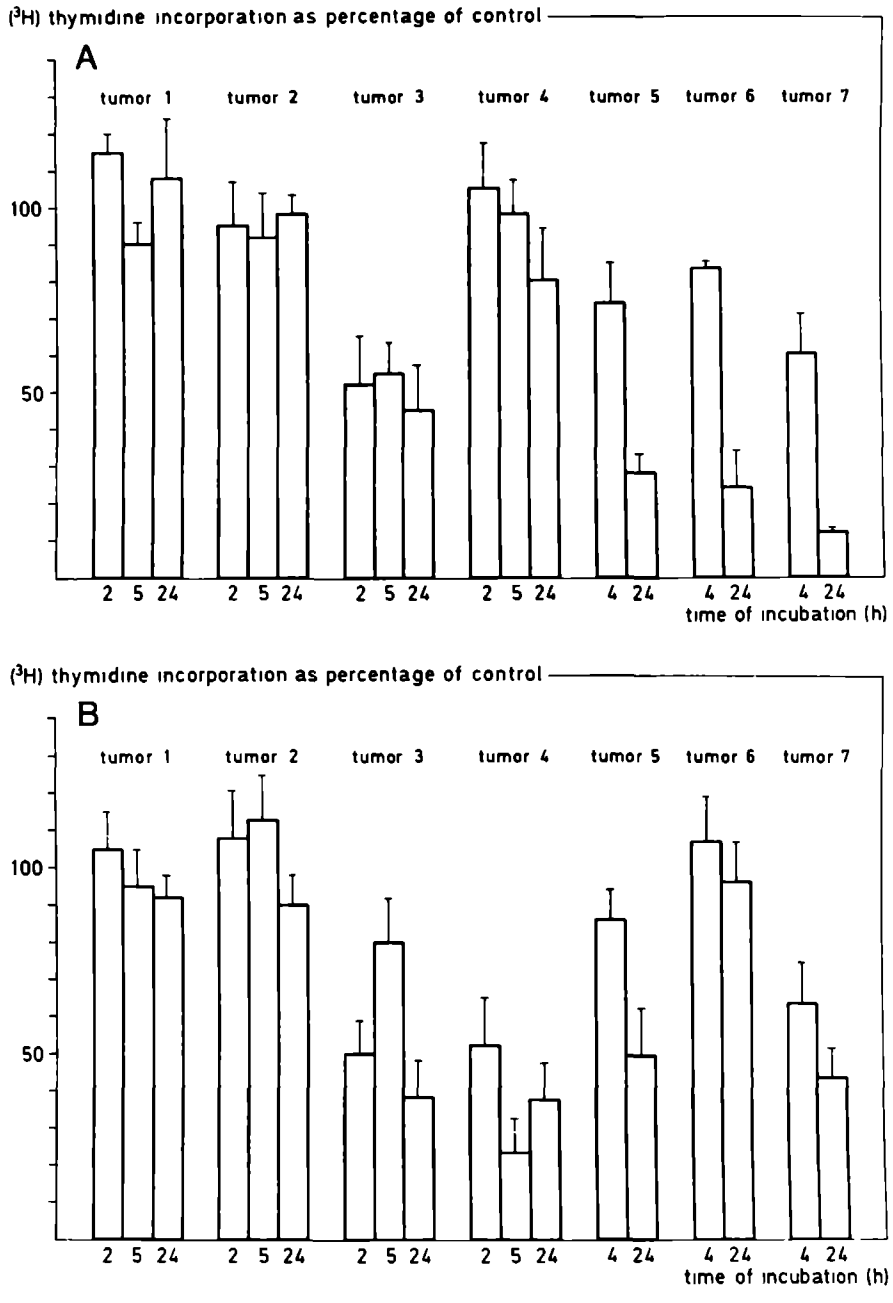

Figure 6.3. Incorporation of ^{3}H-thymidine in slices from seven different squamous cell carcinoma xenografts (see Table 6.I) incubated for various periods in the presence of cisplatin (A) and bleomycin (B). ^{3}H-thymidine incorporation is expressed as a percentage of the control (100%). Each bar represents the mean ± SD of 3 slices. ^{3}H-thymidine was added during the final hour of incubation.

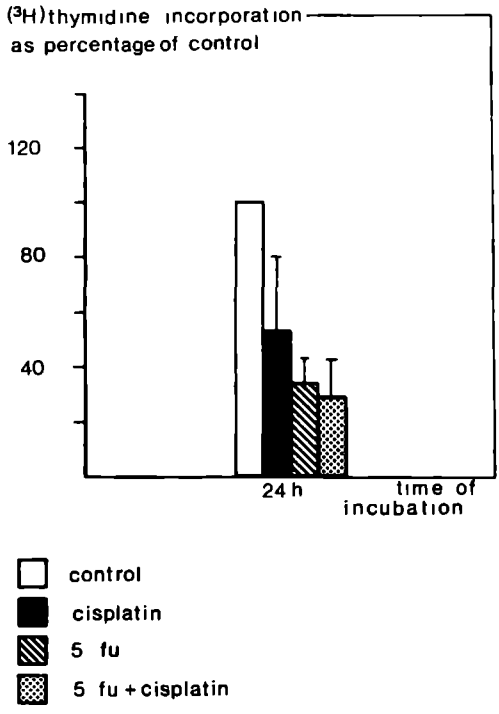

Figure 6.4. Incorporation of ^{3}H-thymidine in slices from a squamous cell carcinoma, directly obtained from a patient, incubated for 24 hours with cisplatin, 5-FU and a combination of cisplatin and 5-FU. ^{3}H-thymidine incorporation is expressed as a percentage of the control (open bar 100%). Each bar represents the mean ± SD of 3 slices. ^{3}H-thymidine was added during the final hour of incubation.

lymph node metastasis (HN-2M) were not sensitive to either bleomycin or cisplatin after an incubation period of up to 24 hours. Tumours HN-4, HN-13 and HN-15 revealed marked inhibition of 3H-thymidine incorporation for both drugs. The sensitivity of tumour HN-6 was limited to bleomycin; no effect could be established for cisplatin. Tumour HN-14 revealed an exclusive sensitivity to cisplatin. Comparison of these data with the effects of cisplatin and bleomycin on the same tumours in nude mice, demonstrated the existence of a close correlation between the results of the in vivo and in vitro chemosensitivity tests (Table 6.I). Only the in vitro sensitivity of tumour HN-14 to cisplatin could not be reproduced in nude mice.
The testing of these tumour lines during different passages failed to show any change in their chemosensitivity features.
So far, the applicability of this in vitro technique to the clinical setting could only be tested on one patient, who had a squamous cell carcinoma of the tongue. Slices of a biopsy of this tumour were incubated either with cisplatin (final concentration 10 mg/ml), 5-FU (5-fluororacil, Abic Ltd., Ramat-Gan, Israel) (final concentration 200 µg/ml) or a combination of both drugs.
Especially 5-FU and the combination of both drugs showed a marked decrease in the incorporation of ^{3}H-thymidine after 24 hours of incubation (Fig. 6.4). The patient was treated with a combination of both drugs and complete regression of the tumour was established after 4 months.

5. Discussion

The use of human tumour xenografts grown in nude mice as a model for testing anti-tumour drugs has several advantages. Apart from the availability of sufficient tumour material for multiple experiments, this approach offers the opportunity to compare the validity of in vitro assays with in vivo testing in nude mice.
From the results obtained in the present study it is obvious that the use of single-cell suspensions is not a reliable technique for chemosensitivity testing, at least for squamous cell carcinomas of the head and neck region. This is especially due to the rapid decrease of ^{3}H-thymidine incorporation during incubation, which reflects a severe loss of cell viability. These findings deviate from comparable studies on several other types of solid tumours, in which single cell suspensions, prepared with similar disaggregation procedures, were employed to study the dose-response relationship of cytotoxic drugs (21, 22). Unfortunately, these studies give no clear impression of the behaviour of these suspensions during incubation, because no absolute figures are given on the uptake of ^{3}H-thymidine.
The discrepancy between cell suspensions of squamous cell carcinoma and other types of solid tumour is most likely due to the peculiar histological architecture of squamous cell carcinomas. Especially the multiple intercellular junctions may be assumed to make the cells more susceptible to irreversible damage by the disaggregation procedures used, which will lead to massive cell death during incubation. This suggestion is supported by the observation that slices of the same tumour, incubated in the same medium, did not show this loss of vitality. Also the

low plating and low cloning efficiency of these tumours in the clonogenic assay (15, 16), seem to be in line with this suggestion.

In contrast to the experience with cell suspensions for cytotoxic drug testing, the results obtained using thin slices are very encouraging. With this method the viability of the cells, based on ^{3}H-thymidine incorporation, could be maintained at a high stable level during an incubation period of up to 30 hours.

The fact that in 6 out of 7 cases close similarity was found between the effects of bleomycin and cisplatin on tissue slices and the same tumours in nude mice supports the validity of this method. The reason for the discrepancy between the cisplatin sensitivity in vivo and in vitro in one of the tumours tested remains obscure.

Although the results of chemosensitivity testing obtained with xenografts cannot simply be transferred to the condition in the patient, a close correlation has been established between the tumour response in the patient and this approach (3, 4, 23).

In summary, this in vitro study demonstrates that the use of slices for the chemosensitivity testing of squamous cell carcinomas, is far superior to the use of single-cell suspensions. It avoids the selection of cells and prevents cellular damage which may lead to serious metabolic disturbances or cell death.

The observations made on slices of xenografts, together with the very accurate prediction of the tumour response with slices of a tumour biopt, strongly suggests that this is a promising technique for chemosensitivity testing. Whether this procedure is suitable for tumour material directly obtained from patients needs further verification on a larger scale.

6. References

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SUMMARY AND CONCLUSIONS

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Over the years both in vivo and in vitro models have been developed to study the biological behaviour of malignant tumours outside the human body. From all these models, the thymus-aplastic nude mouse, which was introduced in the late sixties, has received increasing interest as an in vivo model to study the behaviour of various human malignancies. In the nude mouse, xenografts of many human tumours has been successfully cultured for many generations, generally without significant changes in the original tumour characteristics.
In this thesis, the nude mouse was used as a culture model for human squamous cell carcinomas of the head and neck region.
In chapter I a general review is given of the various in vivo an in vitro models which have been introduced for studying tumour behaviour and cytotoxic drug testing.
Chapter II deals with the transplantability and growth characteristics of head and neck squamous cell carcinomas in nude mice. A high take rate was achieved, while no distinct relationship could be established between the take rate and histological differentation. This score was much higher than the take rate obtained in comparable studies.
The xenografts appeared to retain their original microscopical appearance and degree of histological differentiation. No metastases were found, yet incidentally tumour invasion was observed into the surrounding fibrous capsule.
On the basis of comparisons of growth curves and histological observations, it was concluded that growth curves alone are not a reliable evaluation parameter for tumour growth and the amount of vital tumour tissue present, because of the rapidly increasing accumulation of cellular debris and keratin lamellae within the tumour. As the contribution of non-vital material to the tumour volume only became significant at a tumour diameter of more than 4 mm, the time needed for the tumour to reach a general mean diameter of about 4 mm (initial lag-phase) was chosen as a more reliable parameter for tumour growth.
It is concluded that head and neck carcinomas do not belong to the category of tumours which are difficult to grow in nude mice. The discrepancies between the take rates in the present and other studies are suggested to be due to technical and methodological differences in the grafting procedures.

Besides the maintenance of the original histological features, the preservation of the DNA profile of the donor tumour is of crucial importance to prove that the original clonal composition of the primary tumour has been preserved in the xenograft lines. This is especially important because of the relationship suggested in several studies between DNA ploidy and responsiveness to therapy and the prognosis. Most of the studies have shown that the original DNA profile does not change after xenografting, but some xenografted renal cell carcinomas and osteosarcomas have shown instability of the DNA profile after long-term transplantation in the nude mouse.
The question of the stability of the DNA profile of squamous cell carcinoma of the

head and neck region after xenografting has been addressed in chapter III, with the use of DNA cytometry.
DNA flow cytometry showed an apparent bimodal DNA distribution with an euploid and an aneuploid peak in both the primary tumours and their xenografts. During passaging, the relative proportions of both cell populations could vary, but the DNA indices remained stable. The euploid cell population in tumours with a bimodal DNA distribution is usually assumed to be of host origin, but conclusive evidence is lacking. As routine DNA flow cytometry does not discriminate between mouse host cells and euploid human cells, DNA image cytometry was applied. This technique allows to distinguish between normal host cells and euploid tumour cells. It was clearly shown that the euploid peak represented normal host cells in all the tumours studied. In addition, the mouse orgin of the xenograft stroma was confirmed immunohistochemically with an antibody specifically directed against mouse endothelium.

The preservation of the features of the primary tumour, including the DNA distribution after xenografting, confirms the value of this model for testing cytotoxic drugs. Good correlations have been found in various comparative studies on chemotherapy in patients and xenografts. The evaluation of treatment effects on xenografts has relied largely on changes in the tumour volume, but this method has not yet been standardized. Therefore it was tried to gain more insight into this method by also employing other parameters.
Chapter IV describes chemosensitivity testing of xenografts of squamous cell carcinomas of the head and neck region for cisplatin and bleomycin. These drugs have proved to be effective in patients with head and neck cancer. In addition to changes in the tumour volume, also histology, DNA-flow cytometry and the incidental use of ^{3}H-thymidine were incorporated as parameters for evaluating drug sensitivity.
Three tumours were found to be highly sensitive to both drugs, while only one showed sensitivity to bleomycin. The remaining 4 tumours failed to show any responsiveness during drug treatment.
Comparison of the growth curves and histological sections revealed that the tumour volume alone (growth curves) was not a reliable parameter for measuring the effect of the drug in strongly keratinizing tumours, because of the substantial contribution of the accumulated keratin, which persisted after disappearance of the tumour cells, to the tumour volume.
It is concluded that additional parameters are required for a reliable evaluation of drug sensitivity.
In view of the rapid changes of the DNA profile after drug exposure DNA flow cytometry is suggested as an excellent approach to monitor chemosensitivity.
Regrowth occurred in all the sensitive tumours even after repeated treatment. The recurrent tumour did not show any change in histological features, DNA index or chemosensitivity.

Approaches for predicting drug sensitivity include either xenografts in immunologically incompetent laboratory animals or in vitro tests. The in vivo models can be

assumed to be superior to the in vitro models, where cells are cultured in an artificial environment and the pharmacokinetic influences of the drugs are absent. However, the main disadvantage of in vivo models is that a direct comparison between a xenograft and the primary tumour is difficult, because of the long time needed to establish a tumour line and subsequently chemosensitivity testing. For practical reasons in vitro tests are preferred. Several in vitro predictive tests have been developed for the rapid screening of cytotoxic drug sensitivity, but at present none of these tests have produced satisfactory results.

In Chapter V a new device is introduced allowing the production of sets of slices of equal thickness from tumour xenografts. Incubation of these slices under hyperbaric O_2 showed a reproducible stable incorporation of ^{3}H-thymidine for periods of more than 24 hours. Preliminary data on the effect of cytotoxic drugs showed a close correlation between the in vitro effect and the in vivo sensitivity in nude mice.

Chapter VI deals with two different in vitro techniques for chemosensitivity testing of xenografted head and neck squamous cell carcinomas, using ^{3}H-thymidine as a parameter. Besides the use of thin tumour slices, single cell suspensions were used.

It appeared that in all the cell suspensions of drug-exposed samples and controls, the incorporation of ^{3}H-thymidine showed a fairly sharp decrease during the first few hours of incubation. So no reliable conclusions could be drawn regarding the drug effects.

In vitro incubation of tumour slices in the same culture medium showed the stable incorporation of ^{3}H-thymidine for 30 hours in all the control specimens. Autoradiography revealed ^{3}H-thymidine incorporation into the nuclei of the tumour cells throughout the whole slice. Slices obtained from tumours which were found to be sensitive to cisplatin or bleomycin in vivo in the nude mouse, showed pronounced inhibition of the incorporation of ^{3}H-thymidine when exposed to these drugs. In one tumour, which was found to be insensitive in the nude mouse, in vitro sensitivity for cisplatin was observed.

In addition to these slices of xenografts, slices of one primary tumour were tested. Chemosensitivity testing of slices from this tumour showed close correlation with the patient's response to the same drugs.

The results obtained with the tumour slices seem to be very promising and show that chemosensitivity testing of tumour slices appears to be far superior to the use of single cell suspensions, at least for squamous cell carcinomas.

It is suggested that the disaggregation procedures for obtaining cell suspensions result in irreversible damage to the epithelial cells, leading to cell death. This may be especially marked in epithelial tumours, where the multiple intercellular junctions make the cells more susceptible to damage by the disaggregation procedures. This phenomenon is most probably the main underlying cause of the low plating efficiency encountered in in vitro culturing of these tumour cells.

In summary, the observations made in the present study demonstrate the value of the nude mouse as a reliable in vivo model for studying the biology and chemosensitivity of head and neck squamous cell carcinomas. Although the clinical value of the nude mouse model for the individual patient remains doubtful, the intro-

duction of the tumour slice technique may offer better perspectives for testing drug sensitivity in the individual patient.

SAMENVATTING EN CONCLUSIES

SAMENVATTING EN CONCLUSIES

Voor de bestudering van het gedrag van maligne tumoren buiten het menselijk lichaam zijn zowel in vivo als in vitro modellen ontwikkeld.
Aan het eind van de jaren zestig werd hiervoor de thymusloze, naakte muis geïntroduceerd. Vanaf die tijd is dit in vivo model in toenemende mate in het middelpunt van de belangstelling komen te staan voor het bestuderen van het gedrag van maligne tumoren. Een grote variatie aan maligne humane tumoren is in dit proefdier gedurende vele opeenvolgende generaties gekweekt, zonder dat er significante veranderingen optraden in de oorspronkelijke eigenschappen van de tumoren.

In dit proefschrift wordt een onderzoek beschreven, waarin de naakte muis is gebruikt als model voor het kweken van humane plaveiselcelcarcinomen uit het hoofd-halsgebied.
In het eerste hoofdstuk wordt een overzicht gegeven van de verschillende in vivo en in vitro modellen die in de loop der jaren geintroduceerd zijn voor het bestuderen van het gedrag van maligne tumoren en het testen van hun gevoeligheid voor cytostatica.

In hoofdstuk II worden de resultaten beschreven van een onderzoek naar de transplanteerbaarheid en de groei van plaveiselcelcarcinomen uit het hoofd- halsgebied in de naakte muis. In dit onderzoek werd een hoge take rate bereikt, die veel hoger was dan in vergelijkbare studies en geen relatie vertoonde met de differentiatiegraad van de tumoren. De xenotransplantaten behielden de histologische kenmerken van de oorspronkelijke tumoren. Er werden geen metastasen waargenomen, maar in enkele tumoren werd infiltratie van de tumor in het omringende kapsel gevonden.
Histologisch onderzoek toonde aan dat groeicurves, op basis van het volume van de tumoren, geen betrouwbare weergave vormen voor de groei van de tumoren en de hoeveelheid vitaal tumorweefsel, vanwege de snelle toename van de hoeveelheid keratine en necrotisch materiaal bij tumoren met een diameter groter dan ongeveer 4 mm. Om die reden werd de tijd die nodig was voor de tumoren om een dergelijke diameter te bereiken (initial lag-phase) gekozen als een meer betrouwbare parameter voor de groeisnelheid van deze tumoren.
Uit dit onderzoek werd de conclusie getrokken dat hoofd-halstumoren niet behoren tot de categorie tumoren die moeilijk in de naakte muis getransplanteerd kan worden. De verschillen in take rate met vergelijkbare studies worden toegeschreven aan methodische verschillen en verschillen in transplantatietechniek.

Naast het behoud van de oorspronkelijke histologische kenmerken, is het van essentieel belang te weten of het DNA-profiel van de oorspronkelijke tumor, na xenotransplantatie, ongewijzigd blijft. Hiermee wordt bewezen dat de oorspronkelijke clonale samenstelling van de tumoren in de xenotransplantaten bewaard blijft. Dit is met name van belang, vanwege de relatie die er volgens verschillende onderzoekingen bestaat tussen DNA-ploidy en prognose en de reactie op ver-

schillende therapieën. In het merendeel van de betreffende studies is aangetoond dat het oorspronkelijke DNA profiel van tumoren niet verandert na xenotransplantatie in de naakte muis, alleen bij niercarcinomen en osteosarcomen is incidenteel een instabiliteit in het DNA profiel waargenomen na langdurige passage in de naakte muis.
De stabiliteit van het DNA profiel van plaveiselcelcarcinomen uit het hoofd- halsgebied na xenotransplantatie werd onderzocht m.b.v. DNA flow cytometrie en is beschreven in hoofdstuk III.
De onderzochte primaire tumoren en hun xenotransplantaten vertoonden met DNA flow cytometrie een bimodale DNA verdeling, met een euploide en een aneuploide piek in het DNA histogram. Tijdens de verschillende passages in de naakte muis traden er variaties op in de verhouding van deze twee celpopulaties, maar de DNA indices bleven ongewijzigd. Van de euploide celpopulatie in de xenotransplantaten wordt algemeen aangenomen dat dit normale gastheercellen zijn, maar een experimentele onderbouwing ontbreekt. Met de gebruikte routine DNA flow cytometrie kon geen duidelijk onderscheid gemaakt worden tussen normale muizecellen en euploide humane cellen. Daarom werd gebruik gemaakt van DNA image cytometrie, waarmee deze celpopulaties wel van elkaar kunnen worden onderscheiden. Met deze techniek werd aangetoond, dat de euploide piek in alle onderzochte tumoren uit normale gastheercellen bestond. Daarnaast werd met behulp van een antilichaam, specifiek gericht tegen endotheelcellen van de muis, aangetoond dat het stroma van de xenotransplantaten van de muis afkomstig was.

Het behoud van de kenmerken van de primaire tumor, inclusief het DNA profiel, bevestigt de waarde van dit model voor het testen van cytostatica. In vergelijkende onderzoekingen naar de effecten van cytostatica op verschillende tumoren zijn goede correlaties gevonden tussen de effecten op de primaire tumor en de xenotransplantaten in de naakte muis.
Het meten van de effecten van verschillende therapieën op xenotransplantaten berust grotendeels op veranderingen van het volume van de tumor, maar hiervoor bestaat geen gestandaardiseerde methode. In dit onderzoek werd getracht meer inzicht te verkrijgen in de waarde van deze methode en deze te vergelijken met andere parameters.
Het testen van de gevoeligheid van xenotransplantaten van plaveiselcel- carcinomen uit het hoofd-halsgebied voor cisplatinum en bleomycine wordt in hoofdstuk IV beschreven. Van deze twee cytostatica is bekend dat ze effectief zijn bij de behandeling van deze tumoren. Naast het volume van de tumoren, werden het histologisch beeld, DNA flow cytometrie en bij enkele tumoren de incorporatie van ^{3}H-thymidine als parameters gebruikt voor het meten van de gevoeligheid van de xenotransplantaten voor deze cytostatica. Bij 3 tumoren werd een sterke gevoeligheid gemeten voor zowel cisplatinum als bleomycine, terwijl één tumor gevoelig was voor één van deze stoffen. Bij de resterende 4 tumoren werd geen effect waargenomen. Er kon geen verband worden vastgesteld tussen de differentiatiegraad en de gevoeligheid voor deze cytostatica.
Uit een vergelijking van de groeicurves met het histologisch beeld kwam naar

voren dat de groeicurves in die gevallen, waar de tumoren veel keratine produceerden geen juiste weergave vormden van de mate waarin het cytostaticum effectief was. Dit was het gevolg van het persisteren van keratine na het verdwijnen van de tumorcellen. Op grond van deze waarnemingen werd de conclusie getrokken dat, naast het volume van de tumor, andere parameters nodig kunnen zijn om vast te stellen in welke mate tumoren gevoelig zijn voor behandeling met cytostatica. Op grond van de snelle veranderingen, die na toediening van cytostatica, m.b.v. DNA flow cytometrie in het DNA-profiel van gevoelige tumoren worden waargenomen , wordt deze techniek voorgesteld voor een snelle screening van de effectiviteit van chemotherapie.
Alle xenotransplantaten, die gevoelig waren voor cytostatica, vertoonden hergroei na beëindiging van de therapie. In deze "recidieftumoren" waren na drie achtereenvolgende behandelingen geen veranderingen waarneembaar met betrekking tot de histologische kenmerken, DNA index en gevoeligheid voor cytostatica.

Om de gevoeligheid van tumoren voor cytostatica te testen, worden zowel in vitro cultures als xenotransplantaten in immunologische incompetente proefdieren gebruikt. De in vivo modellen lijken hiervoor meer geschikt dan de in vitro modellen, waar de cellen in een artificieel medium worden gekweekt, waar de pharmacokinetische invloed van de cytostatica afwezig is. Een belangrijk nadeel van de in vivo modellen is echter, dat een directe vergelijking van de donorpatient met het xenotransplantaat erg moeilijk is vanwege de lange periode die er nodig is om voldoende groei van de tumoren te krijgen en deze op hun gevoeligheid voor cytostatica te testen. Om practische redenen wordt daarom de voorkeur gegeven aan in vitro tests. Voor een snelle bepaling van de gevoeligheid van tumoren voor cytostatica zijn er verschillende in vitro tests ontwikkeld maar tot op heden heeft echter geen van deze tests bevredigende resultaten opgeleverd.
In hoofdstuk V wordt een nieuwe in vitro methode geïntroduceerd, waarmee sets van aan elkaar grenzende tumorcoupes, die een gelijke dikte hebben, kunnen worden gemaakt. Incubatie van deze coupes onder hyperbare zuurstof, liet een reproduceerbare stabiele incorporatie van ^{3}H-thymidine zien gedurende een periode van meer dan 24 uur. Een pilotstudie met deze methode naar het effect van cytostatica toonde een nauwe correlatie aan tussen de in vitro effecten en de in vivo gevoeligheid in de naakte muis.
In hoofdstuk VI wordt de vergelijking van twee verschillende in vitro technieken, dunne coupes en celsuspensies, voor het meten van de gevoeligheid van xenotransplantaten van plaveiselcelcarcinomen uit het hoofd-halsgebied voor cytostatica beschreven. Hierbij werd de incorporatie van ^{3}H-thymidine als parameter gebruikt.
Incubatie van celsuspensies van deze tumoren, toonde zowel bij de controle coupes als na toevoeging van cytostatica een sterke afname van de incorporatie van ^{3}H-thymidine aan tijdens de eerste uren van de incubatieperiode. Hierdoor konden geen betrouwbare conclusies getrokken worden over de gevoeligheid van de tumoren voor de toegevoegde cytostatica. Incubatie van tumorcoupes in hetzelfde medium liet daarentegen in alle controle coupes een stabiele incorporatie van ^{3}H-thymidine zien gedurende een periode van meer dan 30 uur. M.b.v. auto-

radiografie kon aangetoond worden dat door de hele dikte van de coupe incorporatie van ^{3}H-thymidine in de tumorcellen was opgetreden. Coupes van tumoren die in de naakte muis gevoelig waren voor cisplatinum en/of bleomycine, vertoonden een sterke remming van de ^{3}H-thymidine opname na toevoeging van deze stoffen aan het incubatiemedium. Deze remming trad niet op bij de ongevoelige tumoren. Bij één tumor echter, waar de opname van ^{3}H-thymidine in vitro duidelijk geremd werd door cisplatinum, kon in de naakte muis geen effect worden vastgesteld.
Naast coupes van xenotransplantaten werden de coupes van 1 primaire tumor met deze methode onderzocht. De in vitro gemeten gevoeligheid voor drie cytostatica correspondeerde met de totale remissie van de tumor, na behandeling van de patient met dezelfde cytostatica.
De resultaten die verkregen zijn met de coupemethode lijken veelbelovend en tonen aan dat in ieder geval voor plaveiselcelcarcinomen het gebruik van coupes als model voor het testen van de gevoeligheid van cytostatica aanzienlijk beter is dan het gebruik van celsuspensies.
De slechte resultaten die met celsuspensies werden verkregen worden geweten aan de disaggregatieprocedures, waardoor de cellen een irreversibele beschadiging kunnen ondergaan en vervolgens snel afsterven. De beschadiging zal speciaal tot uiting komen bij epitheliale tumoren, omdat deze gevoeliger zijn voor disaggregatie, als gevolg van de multipele verbindingen tussen de cellen onderling. Dit is mogelijk ook de oorzaak van de lage "plating efficiency" bij het kweken van deze cellen in vitro.
Samengevat tonen de hier beschreven resultaten aan dat xenotransplantaten van plaveiselcelcarcinomen uit het hoofd- halsgebied in de naakte muis een betrouwbaar model vormen voor de bestudering van het biologisch gedrag en de gevoeligheid voor cytostatica. De klinische waarde van de naakte muis als model voor de behandeling van de individuele patient is echter twijfelachtig. De introductie van de tumor slice techniek lijkt voor het testen van de gevoeligheid van cytostatica betere perspectieven te bieden voor de individuele patiënt.

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CURRICULUM VITAE

De auteur werd geboren op 27 mei 1950 te Djakarta. In 1968 werd het S.M.A.-B diploma te Djakarta behaald. In 1970 werd een aanvang gemaakt met de studie geneeskunde aan de Katholieke Universiteit te Nijmegen. Het doctoraal examen werd in 1976 behaald en het artsexamen in 1978. Vanaf oktober 1978 tot april 1980 was hij als wetenschappelijk medewerker werkzaam op de afdeling Anatomie van de Vrije Universiteit te Amsterdam (Hoofd: Prof. Dr. A.H.M. Lohman). Van 1 april 1980 tot 1 april 1984 volgde hij de opleiding tot keel-, neus- en oorarts in het Sint Radboud Ziekenhuis te Nijmegen (Hoofd: Prof. Dr. W.F.B. Brinkman en Prof. Dr. P. van den Broek). Sedert april 1984 is de auteur werkzaam in het Streekziekenhuis Koningin Beatrix te Winterswijk.